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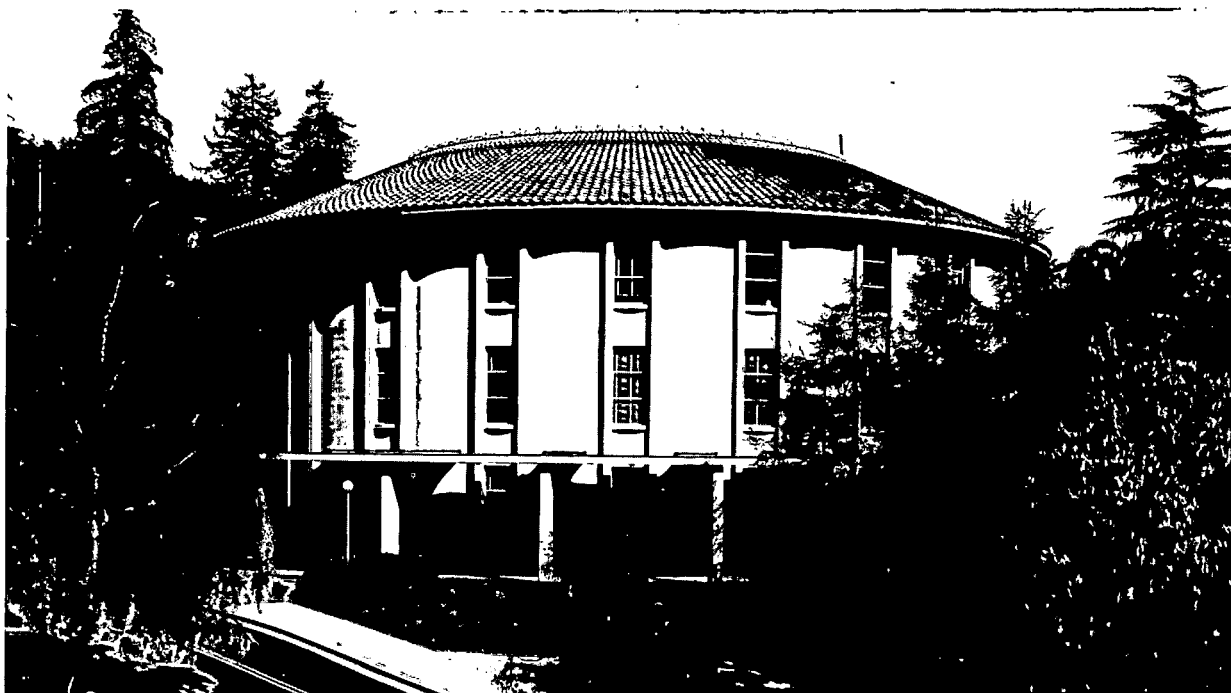
ISOLATION AND CHARACTERIZATION OF PHOTOSYNTHETIC
REACTION CENTERS FROM Rhodospseudomonas capsulata
AND Rhodospseudomonas sphaeroides

S.T. Worland
(Ph.D. Thesis)

September 1984

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ISOLATION AND CHARACTERIZATION OF PHOTOSYNTHETIC REACTION CENTERS
FROM Rhodopseudomonas capsulata AND Rhodopseudomonas sphaeroides

Stephen Theodore Worland

Ph.D. Thesis

September 1984

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Isolation and Characterization of Photosynthetic Reaction Centers
from Rhodopseudomonas capsulata and Rhodopseudomonas sphaeroides

by

Stephen Theodore Worland

ABSTRACT

Reaction centers from Rhodopseudomonas sphaeroides, strain R-26 and Rhodopseudomonas capsulata, strain KZR8A1 were isolated without precipitation by affinity chromatography on equine cytochrome c. Peripheral proteins were removed from the chromatophore membrane by extraction with 0.05% LDAO. After solubilization and adsorption to the cytochrome c column in low ionic strength, the reaction centers were eluted with a non-linear salt gradient. Typically, two passes over a cytochrome c column are required to obtain pure reaction centers. Reaction centers eluted with a non-linear gradient are as pure with respect to polypeptide composition as reaction centers prepared by any of the published procedures. Absorption and EPR spectra and bleaching assays of the primary donor indicate that reaction centers purified by affinity chromatography retain their electron donors and acceptors in the native environment.

The three reaction center polypeptides from KZR8A1 were isolated by preparative electrophoresis and submitted for amino-terminal sequence determination. Exact correspondence is seen between residues assigned by Edman degradation and residues deduced from the corresponding DNA sequences. By comparing the sequences deduced from the DNA to the sequences determined directly from the polypeptides it

was established that the M and L subunits are post-translationally modified to remove the amino-terminal Met, whereas the H subunit is not post-translationally modified at the amino-terminus.

A series of naphthoquinone derivatives was tested for the ability to inhibit O_2 evolution in photosystem II particles from spinach. The naphthoquinone derivatives contain potential leaving groups which confer a latent reactivity to the molecule. Potential inhibitors tested include bromomethyl, acetoxymethyl, and hydroxymethyl derivatives of 1,4-naphthoquinone. The bromomethyl and acetoxymethyl derivatives inhibit O_2 evolution, whereas the hydroxymethyl derivative does not. Inhibition by the acetoxymethyl derivatives is strictly dependent on illumination during the incubation period, suggesting that reduction of the quinone is a necessary prerequisite for inhibition. The inhibition by the acetoxymethyl derivative is not reversible by increased concentrations of electron acceptor. Based on the above observations, it is suggested that acetoxymethyl derivatives of quinones may be useful as suicide reagents capable of labelling quinone binding sites in a variety of quinone binding proteins.

Procedures were developed to extract one or both of the quinones present in reaction centers from KZR8A1. The extraction procedures preserve the integrity of the co-factor binding sites in the reaction center. Reconstitution of quinone-depleted reaction centers with a 10-fold molar excess of ubiquinone resulted in full occupancy at the higher-affinity quinone site and 95-100% occupancy at the lower-affinity site. The higher-affinity site was also reconstituted with 2,3-bis(acetoxymethyl)-1,4-naphthoquinone, a potential suicide

quinone. This compound can accept electrons from the primary donor, as shown by a restoration of the primary donor bleaching.

The H and M subunits were each cleaved at unique sites by treatment with formic acid. Both expected fragments were isolated from the H subunit. Only the larger fragment was isolated from the M subunit. For both subunits, the electrophoretic mobilities of the isolated fragments agrees well with the molecular weights expected from the DNA sequences. The L subunit was digested with Staphylococcus aureus V8 protease. The digestion pattern obtained was consistent with the potential sites of cleavage by the V8 protease, but it was not possible to assign the site(s) of cleavage unambiguously.

Kenneth Sauer

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Several individuals have contributed to the work contained in my thesis. I would like to take this opportunity to acknowledge their efforts and express my gratitude for their help.

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I would like to thank Stephen Isaacs, John Tessman, and David Hearst for the synthesis of the majority of the potential suicide quinones I tested. One derivative was kindly provided by Dr. Alan Sartorelli, Yale.

Sarah Tabbutt first applied the affinity chromatography technique to the isolation of reaction centers from Rps. capsulata, and continually bugged me with lots of intelligent questions.

Trish Maxson helped to develop the procedures for quinone extraction and analysis. Vickie DeRose helped in the large-scale preparations of reaction centers from KZR8A1. Both Trish and Vickie also provided very helpful criticisms of my thesis.

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I would like to thank Dr. John Hearst for enthusiastically welcoming my interest in his research group and for the stimulating discussions concerning the molecular genetics and biochemistry of photosynthesis.

Most of all, I would like to thank my advisor, Dr. Ken Sauer. Throughout my years in his lab he provided a critical analysis of my work and supplied continual encouragement to develop my own scientific interests. I have benefitted greatly from this opportunity to chart my own course of research.

ABBREVIATIONS

ATP	adenosine triphosphate
BChl	bacteriochlorophyll
BPhe	bacteriopheophytin
DTT	dithiothreitol
ENDOR	electron nuclear double resonance
EPR	electron paramagnetic resonance
H	reaction center subunit with the lowest electrophoretic mobility
HPLC	high performance liquid chromatography
L	reaction center subunit with the highest electrophoretic mobility
LDAO	lauryldimethylamine-N-oxide
M	reaction center subunit with an electrophoretic mobility intermediate between that of H and L
NAD ⁺	nicotinamide adenine dinucleotide, oxidized
Q _a	primary quinone acceptor
Q _b	secondary quinone acceptor
<u>R.</u>	<u>Rhodospirillum</u>
RC	reaction center
<u>Rps.</u>	<u>Rhodopseudomonas</u>
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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CHAPTER I: INTRODUCTION

1. GENERAL CHARACTERISTICS OF PHOTOSYNTHETIC SYSTEMS

Photosynthesis is the process whereby living organisms convert electromagnetic radiation into stored chemical potential, and as such, is the fundamental process for the transfer of energy into the biosphere. The energy which is stored is used directly or indirectly by all organisms. The biological importance of the photosynthetic apparatus arises from the dependence of all living organisms on the energy captured during the primary processes of photosynthesis.

Photosynthesis is carried out by a wide variety of organisms, including bacteria, algae, and higher plants. The apparatus used by these diverse organisms to capture light energy are complex and varied, but an underlying similarity can be seen despite the diversity. A general description will be provided of the features common to all photosynthetic organisms, followed by a more detailed description of the apparatus of purple non-sulfur bacteria.

In all photosynthetic organisms initial absorption of a photon occurs in pigment-protein complexes. The pigments found in photosynthetic systems have absorption maxima ranging from the near ultraviolet (350 nm) into the infrared (1000 nm). The pigment-protein complexes found in a particular organism have absorption spectra which are coincident with the light wavelengths found in their native habitats. This represents an evolutionary adaptation of the photo-synthetic organisms to the physical environments in which they exist (Glazer, 1980). The absorption spectra of pigments are often modified by complexation with the

protein. The existence of pigment-protein complexes in which the absorption spectrum of the complex, not the isolated chromophore, matches the incident radiation reflects co-evolution of the pigment biosynthetic pathway and the structural proteins of the light-absorbing complex.

Typically photons are initially absorbed by antenna systems which transfer the excitation energy to a complex known as a reaction center (RC, see below). The physics of excitation transfer has been discussed recently (Pearlstein, 1982). The salient features will be reviewed here. The basic mechanism of excitation transfer involves coupling between the electronic and vibronic excited states of the chromophores. It is the magnitude of this coupling which determines the rate of excitation transfer. The older photosynthetic literature contains many references to two "mechanisms" of excitation transfer, exciton coupling and Förster transfer. Pearlstein emphasizes that this is an inappropriate division. The mechanism of excitation transfer is always the same, i.e. coupling between electronic and vibronic excited states. The time development of excitation transfer shows two distinct regimes. The characteristics of excitation transfer within each of these time domains are similar to the characteristics which have been attributed to the two "mechanisms" of excitation transfer.

On a very short time scale, 10 fs to 1 ps, phase coherence exists between the excited states of the chromophores. Excitation transfer during this time regime has a wavelike nature. The excitation residence time is so short that the excitation can be thought of as being distributed among all the chromophores

simultaneously. The excited state is really a state of the whole array of chromophores. After approximately 1 ps (the exact time here is subject to controversy) phase coherence between the excited states is lost. The excitation residence time is now long enough that the excitation can be thought of as "hopping". The hopping can be from one molecule to another molecule, or from a group of very closely spaced molecules (e.g. the chromophores contained in one protein complex) to another group of closely spaced molecules. The rate of hopping varies inversely with the sixth power of the distance between the chromophores, and varies directly with the overlap between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. The hopping rate varies inversely with the radiative lifetime of the excited state, and depends on the relative orientation of the chromophores. Excitation transfer from one pigment-protein complex to another complex occurs on a time scale greater than 1 psec, and thus it is appropriate to think of excitation "hopping" from complex to complex. Excitation transfer within one complex is probably fast enough (< 1 psec) to be at least partially coherent.

Excitation energy is ultimately transferred from the antenna to a complex known as a reaction center. The reaction center is the site where electromagnetic radiation is converted into chemical potential. All reaction centers consist of a protein matrix to which are bound various cofactors. The cofactors found include bacteriochlorophyll [(B)Chl], bacteriopheophytin, [(B)Phe], quinones, and iron-sulfur clusters. For a general review of reaction center structure and composition see (Okamura et al,

1982a). The conversion of photon energy to stored chemical energy is accomplished by using the energy of a photon to drive an electron transfer reaction. The primary reductant is an electronically excited state of (B)Chl. Electron transfer from the ground state of the (B)Chl species is highly endergonic. The electronically excited state, however, has a much lower midpoint reduction potential than does the ground state. In other words, an electron is much less tightly bound in the excited state than in the ground state. The loosely bound electron is transferred from the excited (B)Chl to a nearby acceptor, in an exergonic reaction. The extended π electron cloud of (B)Chl is particularly well suited for the role of primary electron donor (Mauzerall, 1978). In bacteria the primary donor is thought to be a dimer of BChl, as first suggested by Norris and coworkers (Norris et al, 1971). Donation of an electron from the primary donor will leave an unpaired electron behind.

Delocalization of this unpaired electron over the conjugated systems of two (B)Chl's would further reduce the energy needed to donate an electron. A more detailed description of the structure of reaction centers from purple, non-sulfur bacteria will be provided below.

Fig. I-1 shows the sequence of primary electron transfer events within a bacterial reaction center (taken from Parson and Ke, 1982). This figure can serve as a general model for the discussion to follow. The first electron acceptor must be a reducible molecule which is positioned within the reaction center protein close to the primary donor. Upon reduction of the first acceptor, the energy of the photon which created the electronically excited state of (B)Chl is converted into the chemical potential of a redox couple. The

Fig. I-1. Electron transfer kinetics in reaction centers from
Rps. sphaeroides, strain R-26.

Electron Transfer Kinetics

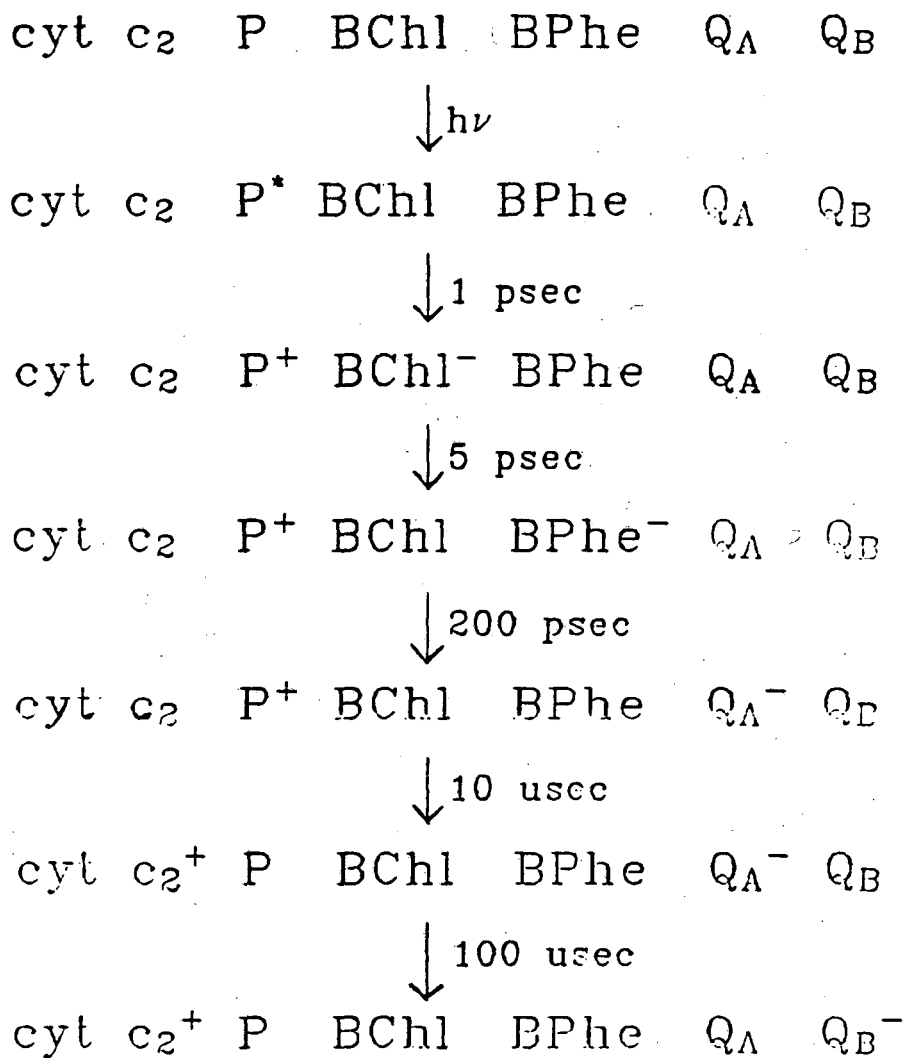


Fig. I-1

initial electron transfer reaction creates a radical-pair state (Parson and Ke, 1982). This radical-pair state potentially has a high probability of back reaction, leading to loss of the energy captured during electron transfer. In photosynthetic membranes the extent of wasteful back reactions is minimized by positioning a series of electron acceptors after the initial acceptor. Several electron transfer steps occur. Each step has a high probability of forward reaction and a lower probability of back reaction. Several such electron transfer reactions, placed in series, result in a high probability of forward electron flow while greatly reducing the chance of reverse electron flow. In this manner a portion of the chemical potential stored in the first electron transfer reaction is conserved for use by the organism.

Parson has discussed the qualitative requirements for maximizing the rate of forward electron transfer while minimizing the rate of reverse transfer (Parson and Ke, 1982). More physically detailed models, emphasizing the importance of overlap between allowed nuclear vibrational modes of donors and acceptors, have also been presented (Jortner, 1980, Hopfield, 1979). In Parson's description, electron transfer rates are a function of orbital overlap between donor and acceptor molecules. Referring to Fig. 1, fast forward electron transfer will be favored by a strong orbital overlap between P^* and BPhe. Slow back transfer will be favored by weak overlap between P^+ and $BPhe^-$. Similarly, efficiency of energy conversion will be increased by strong orbital overlap between $BPhe^-$ and Q_a and by weak overlap between $BPhe$ and Q_a^- . The extent of orbital overlap depends on the molecules involved, as well as the

occupancy of the orbitals involved. It is partly the difference in occupancy that permits different orbital overlap in the states P^+BPhe and P^+BPhe^- . Interactions between the protein and the electron donors and acceptors may also affect the extent of orbital overlap. The initial electron donors and acceptors are bound tightly to the protein matrix of the RC. The binding forces can involve dipolar interactions, hydrogen bonding, and direct orbital overlap between the donors or acceptors and certain amino acid residues (eg. His, Met, Tyr, Phe, Ser). Such interactions with the protein can alter the orbital overlap between different cofactors, and in this way directly affect the rates of electron transfer. For this reason any detailed explanation of the efficiency of photosynthetic energy conversion requires knowledge of the interactions between the protein and cofactor portions of the reaction center.

Recent experimental results indicate that the above description may place too much emphasis on direct orbital overlap. Efficient photo-induced electron transfer has been observed in synthetic porphyrin-quinone systems in which the electron donors and acceptors cannot experience orbital overlap (Joran et al, 1984; Wasielewski et al, 1984). The synthetic models used in these studies contain a rigid linker between the porphyrin and quinone, excluding the possibility of direct orbital overlap between electron donor and acceptor. The fact that efficient electron transfer is still observed indicates that orbital overlap is not a requirement for efficient electron transfer. Within the RC, any space between electron donor and acceptor is likely to be occupied by amino acid

side groups. Interactions between the side groups and the donors or acceptors may have an important role in determining the rates of electron transfer and the unidirectional nature of electron transfer.

Electrons are eventually transferred out of the RC, to small electron carriers (eg. quinones) and to other protein complexes (eg. cyt bc_1 or b_6f complex). Most of the electron accepting species are embedded in a lipid bilayer (see Kaplan and Arntzen, 1982, for a general review). The organization of the complexes within the membrane is important because it permits electron transfer reactions to be coupled to a vectorial transport of protons (see below). Photosynthetic membranes have a very high protein:lipid ratio. In photosynthetic bacteria, this ratio is typically 3:1 (w:w) but can be as high as 5:1 prior to cell division (Fraley et al, 1979a). Such a high protein content can have important effects on the fluidity of the membrane (Fraley et al, 1979b). The pebble mosaic model of a photosynthetic membrane (Sauer, 1978) suggests a lower lateral mobility of photosynthetic complexes as compared to complexes in less protein-rich membranes.

The electron donating and accepting complexes are inserted asymmetrically in the lipid bilayer. Electron transfer within a given complex, and between complexes, generates a shift in the absorption spectrum of carotenoids in the membrane (see Junge and Jackson, 1982). This band shift, due to the Stark effect of an electric field, demonstrates the electrogenic nature of electron transfer in the photosynthetic membrane. Because the proteins are inserted into the membrane in a defined, not random, orientation,

electron transfer within several complexes can lead to the accumulation of a substantial electric field across the membrane. A pH gradient is also established across the membrane as a result of electron transfer. This pH gradient can be demonstrated using dyes whose absorption spectra are a function of pH.

Bacterial RC's and photosystem two (PSII) of higher plants both contain quinones as electron acceptors. Oxidation of (B)Chl involves only electron transfer, but quinone reduction involves hydrogen atom transfer (the electron and proton transfer need not occur simultaneously). Reduced quinones are reoxidized at the cyt bc_1 complex (b_6f in chloroplasts). Reduction of the cytochromes involves transfer of electrons, while oxidation of the dihydroquinone involves abstraction of hydrogen atoms. The chemiosmotic theory (Mitchell, 1966) proposes that the protons needed for quinone reduction come from one side of the membrane, while the protons abstracted in hydroquinone oxidation are released on the opposite side of the membrane. In this way an electron transfer pathway which contains alternately electron carriers (protein-bound redox clusters) and mobile hydrogen atom carriers (quinones) can be utilized to generate a pH gradient. The chemiosmotic theory has two prerequisites. First, the sites of quinone protonation and deprotonation must be located on opposite sides of the membrane. Second, quinones must be able to diffuse in the membrane fast enough to match the measurable rate of ATP synthesis (a few msec after the onset of illumination, Ort et al, 1976, Vinkler et al, 1980). Neither of these requirements of Mitchell's theory have been experimentally verified. However, it has been is experimentally

verified that electron transfer generates a pH gradient, and the chemiosmotic theory suggests an attractive explanation.

The pH gradient generated by electron transport is used to synthesize ATP. The ATP synthesizing enzyme, referred to as ATPase or coupling factor ($CF_0 + CF_1$) consists of two main subassemblies (McCarty and Carmeli, 1982). The membrane-bound portion, CF_0 , is a channel through which protons can pass. The water-soluble portion, CF_1 , contains the site of ATP synthesis. Many of the details whereby the energy stored in a pH gradient is used to synthesize ATP are not known. Particularly, it is not known whether the pH gradient is established in the bulk aqueous phases on either side of the membrane or is localized in an aqueous layer associated with the water/lipid interface. Also unknown is the enzymatic mechanism whereby the high energy phosphate bond is synthesized.

Besides ATP synthesis, photosynthetic organisms utilize light-driven electron transfer to generate reducing equivalents. Two fundamentally different mechanisms are used by different classes of photosynthetic organisms. Purple, non-sulphur bacteria contain a cyclic electron transfer pathway. At no point in this pathway is a stable reductant generated with a potential low enough to reduce NAD^+ ($E_m' = -320$ mV). These organisms contain an ATP dependent NAD^+ reductase. The electrons for this reduction come from organic substrates extracted from the environment, while the energy for the endergonic transfer of electrons from substrate to NAD^+ comes from the hydrolysis of ATP. Green sulphur bacteria, algae, and higher plants contain a linear electron transfer pathway. These organisms reduce $NAD(P)^+$ as the terminal step in the light-driven electron

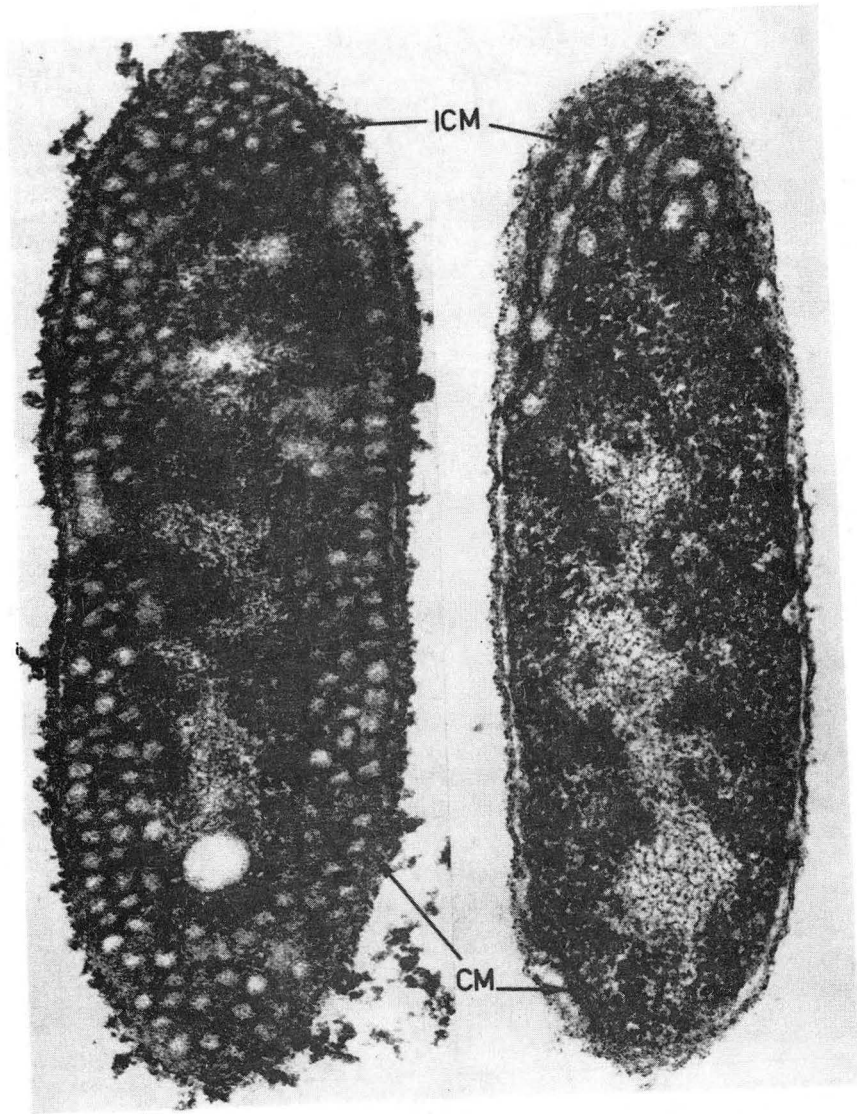
transfer pathway. Electrons are continuously fed into the other end of the pathway, from H_2S or H_2O . In both classes of organisms, reducing equivalents are generated for use in the anabolic reactions of the organism.

2. PHOTOSYNTHETIC APPARATUS OF Rhodopseudomonas capsulata AND Rhodopseudomonas sphaeroides

The photosynthetic apparatus of Rhodopseudomonas (Rps.) capsulata and Rps. sphaeroides is contained within an extensive intracytoplasmic membrane. This membrane structure is almost absent in Rps. capsulata cells grown aerobically (see Fig. 1-2 from Lampe et al, 1972). In Rps. sphaeroides the intracytoplasmic membrane is quantitatively absent in aerobic cells. When the O_2 tension is lowered below a species-specific level, the extensive intracytoplasmic membrane seen in Fig. 1-2 develops. Both ultrastructural and biochemical data suggest that the intracytoplasmic membrane is continuous with the cytoplasmic membrane, although the protein compositions of the two membranes are different (Kaplan and Arntzen, 1982). Furthermore, all of the periplasmic face of the intracytoplasmic membrane is accessible from outside the cell, as shown by chemical labelling experiments (Kaplan and Arntzen, 1982, p. 97). The mechanism for maintaining separate domains in a physically continuous membrane structure is not known.

The intracytoplasmic membrane has a very high protein/lipid ratio. This high protein content is not constant, however. By using synchronously dividing cultures, it was shown that the protein/lipid ratio (w/w) can be as low as 2.0 immediately following

Fig. I-2. Electron micrograph of cells from Rps. capsulata.
ICM-intracytoplasmic membrane. CM-cytoplasmic membrane. Left,
anaerobically grown cell. Right, aerobically grown cell.



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Fig. I-2

cell division, and as high as 5.0 prior to cell division (Fraley, 1979 a). The rate of protein insertion into the membrane is constant during the cell cycle, but the rate of lipid insertion varies in a step-wise fashion, resulting in the different protein/lipid ratios.

The intracytoplasmic membranes are typically isolated from French press effluents by ultracentrifugation. The membrane fractions appear as vesicles 60-90 nm in diameter. By the use of gel filtration chromatography (Fraker and Kaplan, 1971) vesicular membranes can be obtained which are virtually free of contamination by ribosomes, cytoplasmic membranes, or cell wall debris. The vesicular membranes obtained by ultracentrifugation are typically referred to as chromatophores.

Chromatophores contain all of the components of the photosynthetic apparatus necessary to generate a membrane potential and pH gradient in the light, including light-harvesting proteins, the reaction center, cytochrome bc₁ complexes and ubiquinones. In chromatophores from wild type cells of Rps. sphaeroides or Rps. capsulata, one finds two broad classes of light-harvesting complexes (Cogdell and Thornber, 1980). The B875 complex contains two polypeptides, BChl, and carotenoid (crt). Within the chromatophore membrane, this complex is probably aggregated. The amino acid sequences of both B875 polypeptides are known (Youvan et al, 1984a, Zuber et al, unpublished). A common feature of the sequences of LH proteins from many species of Rhodospirillaceae is a His residue in the middle of a hydrophobic sequence of amino acids. Zuber and coworkers suggested that this His is the binding site for BChl

(Tadros et al, 1984), based on the known structure of a water soluble Chl protein (Mathews et al, 1979) in which Chl is ligated to His.

The B800-850 complex from Rps. capsulata contains 3 polypeptides, the smaller two of which bind BChl and crt (Feick and Drews, 1978). The same complex from Rps. sphaeroides was long thought to contain only two polypeptides (Cogdell and Thornber, 1980). Recent results indicate the existence of a third polypeptide (Cohen and Kaplan, 1981) which may be a component of the B800-850 complex. There is also evidence which suggests involvement of another smaller polypeptide in the Rps. sphaeroides complex (Kiley and Kaplan, 1984). The B800-850 complex has been isolated in aggregated form, as a complex of MW 200 kD (Shiozawa et al, 1982). This unit was suggested to be the aggregated form found in vivo, but this is rather tentative. A common problem with the isolation of LH proteins is relating the state of assembly found in the purified complex to the native state of assembly in the membrane. For the LH complexes isolated from Rps. sphaeroides and Rps. capsulata this problem has not yet been adequately addressed.

Excitation energy from the light-harvesting complexes is transferred to the RC, where the photochemical reaction takes place. Structural details of the RC are provided in the last section of the introduction. The last RC-bound acceptor in the electron transfer path is a quinone (see Fig. 1). The singly reduced quinone remains bound to the RC, in the form of a radical anion (Wraight, 1977). Further reduction of the quinone, by a second electron from the primary donor, is followed by uptake of two protons (Wraight, 1979).

The dihydroquinone is free to diffuse away from the RC. This leaves the Q_b site vacant, ready to be occupied by another oxidized quinone.

Several phenomena associated with the acceptor side of bacterial RC's and PSII from higher plants show a period two oscillation when probed with flashing light. Among these phenomena are absorption changes associated with formation of a semiquinone anion (Wraight, 1977, Vermeiglio, 1977), herbicide binding (Laasch et al, 1983), and availability of electrons from PSII to re-reduce oxidized PSI (Bouges-Bocquet, 1973). Wraight and Vermeiglio independently proposed a two-electron gate model to explain these observations. The two-electron gate model proposes that quinone and dihydroquinone are bound only weakly to the quinone binding site, while the semiquinone anion is bound very tightly. By binding to the RC the semiquinone is stabilized long enough for a second electron to arrive from the primary donor. In this manner the one-electron oxidation of (B)Chl is coupled to the two-electron reduction of quinone. The two-electron gate model suggests that herbicides are able to displace quinone or dihydroquinone from the Q_b site, but unable to displace semiquinone. This gives rise to the oscillatory nature of herbicide binding.

Interactions with the RC protein are probably responsible for the greatly increased stability of the semiquinone when bound to the Q_b site. Regions of homologous amino acid sequence are seen between two subunits of the bacterial RC (referred to as the M and L subunits) and the Q_b -binding protein of higher plants (Williams et al, 1983; Youvan et al, 1984b; and Zurawski et al, 1982). Hearst

and Sauer (1984) proposed an interaction between the quinone and the amino acid residues common to M, L, and the Q_b protein. The homologous sequences are primarily hydrophobic, except for His and Met residues. It was suggested that the His and Met are responsible for binding quinones and providing the stabilization of the semiquinone form of Q_b . Azido-atrazine, an analogue of the herbicide atrazine, forms a covalent attachment to the Q_b -binding protein upon UV illumination (Pfister et al, 1981 and Gardner, 1981). The site of covalent attachment has been tentatively assigned to an 85 amino acid tryptic fragment of the Q_b protein (Wolber and Steinback, 1984). This 85 amino acid fragment contains the sequences homologous to the M and L subunits.

Dihydroquinones are reoxidized at the quinol-cytochrome c oxidoreductase (bc_1 complex). This complex is similar in many respects to Complex III in mitochondria and the quinol-plastocyanin oxidoreductase (b_6f complex) in higher plants (Hauska et al, 1983). The bc_1 complex contains a c-type cyt, 2 b-type cyts, and a Rieske iron-sulfur center. Oxidation of quinones by the bc_1 complex results in reduction of the hemes and iron sulfur components of the bc_1 complex. As yet the exact sequence of these reductions is not determined, but it is believed that electrons pass through the iron-sulfur center to the cyt c_1 and on to a water-soluble cyt c_2 .

The water-soluble cyt c_2 which is reduced by the bc_1 complex reduces the oxidized primary donor in the RC. This completes the cyclic electron transport pathway which was initiated by photooxidation of the primary donor. Antibody labelling studies have shown that cyt c_2 is located within the periplasmic space

(Prince et al, 1975). The cyt c_2 binding sites on both the RC and bc_1 complex must therefore be located near the periplasmic side of the chromatophore membrane, or extended into the periplasmic space. Cross-linking studies suggest that in bacterial RC's the cyt c_2 binding site is contained on the M and L subunits (Rosen et al, 1983). This indicates that M and L should be near, or protrude into, the periplasmic space. The cyt c_2 binding site on the bc_1 complex has not been identified by crosslinking, but electrons are thought to pass from the Rieske Fe-S center through cyt c_1 to cyt c_2 . This suggests that the cyt c_1 protein is close to the periplasmic space.

In chromatophores, the stoichiometry of protons translocated across the membrane per electron transferred through the chain can be greater than one under some conditions (Hauska et al, 1983). For this system, a proton/electron (p/e) ratio greater than one is inconsistent with Mitchell's original formulation of the chemiosmotic model (Mitchell, 1966) in which each protein complex in an energy transducing membrane has only one quinone binding site. Mitchell proposed the "Q loop" to explain a p/e ratio greater than one (Mitchell, 1976). This model has been extended to Rps. capsulata and Rps. sphaeroides (Crofts et al, 1983). The Q loop proposes two quinone binding sites on the bc_1 complex. At the first quinone binding site quinones which were reduced by the RC are reoxidized. One electron from each quinone is passed through the Rieske Fe-S center and through cyt c_1 to cyt c_2 . The other electron is passed through the b cyt's to a second quinone binding site, located near the cytoplasmic side of the membrane. A quinone bound

at this site is stabilized in the semiquinone form until another electron arrives from the first quinone site. Recently, an EPR signal similar to what is expected from a semiquinone has been detected in the isolated bc_1 complex (de Vries et al, 1982). This semiquinone may be bound at the second quinone binding site proposed by the Q loop model. The model predicts that full reduction of a quinone at the second binding site is accompanied by binding of two protons from the cytoplasmic side of the membrane. Oxidation of this quinone at the first binding site results in a p/e ratio greater than 1. An alternative sequence of electron transfer steps, called the "b loop model", proposes that the b cyt's are in a linear sequence of electron carriers (Wikstrom et al, 1981). In this model, the proton/electron ratio is explained as resulting from a conformational change in the protein of the b cyt, resulting in release of protons to the periplasmic space. The b loop model does not require an integral p/e ratio, because the number of protons released from the protein need not have any direct relationship to the number of electrons transported. To date, no one has demonstrated more than one quinone binding site on the bc_1 complex, nor has anyone demonstrated a conformationally induced release of protons from the proteins of the b cyt's. Therefore, no conclusions can be drawn concerning the validity of either model.

3. STRUCTURE OF REACTION CENTERS FROM Rhodopseudomonas sphaeroides AND Rhodopseudomonas capsulata

Bacterial RC's were first isolated to near homogeneity independently by Feher (Feher, 1971) and Clayton (Clayton and Wang,

1971). RC's were isolated from the R-26 mutant of Rps. sphaeroides. This mutant lacks colored carotenoids and has an altered light-harvesting protein composition (Clayton and Smith, 1960). Both methods involve mild detergent extraction of the RC's from the chromatophore membrane followed by fractionation with ammonium sulfate. Further purification was obtained using ion-exchange chromatography. A detailed protocol for this method is available (Feher and Okamura, 1978, appendix). The most important result of these two isolations was the preservation of light-induced charge separation in a fairly small complex; a complex small enough that its composition could reasonably be determined.

RC's have been isolated from carotenoidless mutants of Rps. capsulata (Prince and Crofts, 1973 and Nieth et al, 1975) using procedures similar to those used with strain R-26. The Rps. capsulata RC's obtained by both groups have a near-IR absorption spectrum somewhat different than that of RC's from R-26. In their preparations from Rps. capsulata, the lowest-energy absorption band is at 850 nm, while it is at 865 nm in RC's from Rps. sphaeroides, strain R-26. Also, the ratio of A_{760}/A_{850} is 1.3 in the Rps. capsulata RC's, but the ratio A_{760}/A_{865} is 1.0 in RC's from strain R-26. In Chap. II I will present evidence which suggests that the different position of the lowest-energy band is a true species difference, not a consequence of detergent solubilization. The relatively high absorption at 760 nm is probably related to the isolation procedures, because this high absorbance is not seen in the RC's prepared in this work.

The absorption spectrum of RC's contains three maxima in the

near IR, at approximately 760, 800, and 850-865 nm. The position of the lowest energy maximum is different depending on the organism and possibly the isolation procedure. This is discussed more fully in Chap. II, part 3. The lowest energy band is bleachable in strong (10 mW/cm^2) actinic light. This bleaching is a consequence of electron donation from the primary donor to an acceptor.

Preservation of reversible photobleaching of the 850-865 nm band is a benchmark requirement of any reaction center isolation.

Essentially, bleaching is the assay of enzymatic activity, which in this case is light-induced charge separation. Other assays include reversible generation of an EPR signal at $g=2.003$ due to oxidation of the primary donor, and reversible generation of an absorbance change at 450 nm due to reduction of the quinone acceptor.

Bleaching at 850-865 nm is the most convenient assay, because the extinction coefficient is large ($1.1 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, Straley et al, 1973) and the only instrumentation required is an absorption spectrophotometer equipped for side illumination. Bleaching at 850 or 865 nm was used to assay RC's for the work in this thesis, although generation of a photo-induced EPR signal was demonstrated as well. The absorption spectrum of RC's also contains maxima in the visible and UV regions, attributed to the Qx and Soret bands of BChl and BPhe. RC's also absorb light near 280 nm, due primarily to protein. Ubiquinone also contributes a small intensity at 280 nm. In a carotenoidless mutant of Rps. capsulata, additional absorbance in the UV may be due to a carotenoid precursor (see Chap. II, part 3).

The absorption spectrum of RC's indicates the presence of BChl and BPhe. The exact pigment composition was determined by

extracting the pigments into organic solvents and comparing the resultant spectra to known standards (Straley et al, 1973). The pigment composition was put on a molar basis by following the rereduction of the primary donor by cyt c_2 . (The oxidized minus reduced extinction coefficient of cyt c_2 was already known). These experiments indicated that one mol RC contains 4 mol BChl and 2 mol Bphe.

RC's isolated from carotenoid-containing strains have a bound carotenoid (Jolchine and Reiss-Husson, 1974). After isolation, RC's from R-26 will bind all-trans sphaeroidenone in approximately a 1:1 molar ratio to P870 (Agalidis et al, 1980). Resonance Raman spectra indicate that binding to the protein induces an isomerization from all-trans to at least one cis bond.

The presence of ubiquinone (UQ) in RC's was established by extraction and reconstitution experiments (Cogdell et al, 1974). Extraction of all UQ's abolished photochemistry as measurable with a standard side illumination setup. Charge separation can still occur in RC's lacking quinones, but the back reaction is so fast (10 ns) that detection is very difficult. The number of UQ binding sites in R-26 RC's was determined by extensive reconstitution experiments (Okamura et al, 1975). There are two binding sites for UQ in the RC. One binding site has a dissociation constant of approximately 1 μM , the other site has at least a five fold lower affinity (Okamura et al, 1982b). Electron transfer is sequential, from the tightly bound UQ to the loosely bound UQ. Upon reduction, a quinone at the Q_b site is stabilized until a second electron arrives from the primary donor. In vivo, electrons are restored to the oxidized RC

by donation from cyt c_2 . Binding of one UQ to the RC (at the high affinity site) is sufficient to fully restore the readily observed charge separation. In vitro, a UQ/RC ratio of 10 (mol/mol) is required to occupy fully the second UQ binding site. In the chromatophore membrane the UQ/RC ratio is 10-20, but UQ is much more soluble in the lipid bilayer than in the detergent solution used to make the in vitro binding measurements. In vivo, a fraction of RC's may have an unoccupied Q_b site.

Atomic absorption spectra indicate the presence of Fe in RC's. Depending on the composition of the growth medium, some of the Fe can be replaced by Mn (Feher et al, 1974). Mössbauer spectra and magnetic susceptibility studies indicate that the Fe(II) does not change valence during photo-oxidation of the primary donor (Butler et al, 1980). These studies also show that the zero-field splitting parameters for the Fe are unchanged upon removal of Q_b , suggesting that Q_b is not a ligand to the Fe. The iron does interact with the reduced acceptors. The EPR signal of reduced Q_a or Q_b shows magnetic coupling to the Fe (Feher et al, 1974). Because of the similarities in the EPR spectra and relaxation times when the electron resides on Q_a or Q_b , the Fe is thought to be approximately equidistant from both quinones (Butler et al, 1984). The similarities in the spectra also indicate that, since Q_b is not a ligand to the Fe, Q_a is probably not a ligand either.

The primary photochemistry in the RC generates unpaired electron spin density on both the donor and acceptors. As a consequence, EPR and ENDOR spectroscopies have been very useful, both as a way to elucidate structural information and as a method of

following the sequence of electron transfer reactions. The primary donor in bacterial RC's has a characteristic radical EPR signal, centered at $g=2.0026$, which is 9.8 G wide at 77 K (Feher et al, 1975). This signal is approximately 1.4 times more narrow than that of $BChl^+$ in solution, which led to the proposal that the primary donor was a BChl dimer (Norris et al, 1971). Sharing of the unpaired electron density over two conjugated ring systems is expected to decrease the signal width by a factor of about 1.4. A problem with this interpretation is that the EPR signal from the primary donor is inhomogeneously broadened, making interpretation of the width of the signal difficult. In the near future, application of spin echo EPR may allow the resolution of sharper signals which are only homogeneously broadened. Much more structural information is potentially available from these signals.

The coupling between the unpaired electron spin and the protons of the primary donor has been investigated by ENDOR. Coupling between the electron and the proton splits the proton NMR spectrum into a doublet. The magnitude of the splitting is sensitive to the the electron spin density interacting with each proton. The ENDOR spectrum of the primary donor shows splitting only half as large as seen in $BChl^+$ in solution (Feher et al, 1975) Again, this is consistent with the unpaired electron being shared over two BChl molecules.

The ENDOR results are often interpreted as being a definitive assignment of the primary donor as a BChl dimer. This is not really valid, because the electron density was sampled only at two of the protons on the BChl molecule. An alternative explanation of the

ENDOR spectrum has been put forward (Babcock et al, 1984). They postulate that interactions between the protein and the primary donor lead to a mixing between the ground state of $BChl^+$ and the first electronic excited state of $BChl^+$. This mixing is seen as the cause of the altered electron spin density interacting with the protons observed in the ENDOR spectrum. (Reasonable molecular orbital calculations for the ground and excited states of $BChl^+$ are available, so one can calculate the electron spin density interacting with a given proton). An attractive feature of this model is that the mixing deduced from the ENDOR data predicts nearly quantitatively the lowest energy absorption band seen for the primary donor (1250 nm for the primary donor versus 950 nm for $BChl^+$ in solution).

Pearlstein has also raised objections to the $BChl$ dimer model for the primary donor (Pearlstein, 1982). The objections are based on the positions, linewidths, and relative intensities of transitions seen in the optical and circular dichroism spectra of RC's from R-26. Pearlstein argues that these effects are best explained by assuming that the absorption band at 865 nm arises from one $BChl$ (the primary donor molecule), and that the absorption band at 800 nm arises from the other three $BChl$ in the RC.

The polypeptide composition of RC's was investigated by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). Using the Laemmli buffer system (Tris/glycine, Laemmli, 1970), three polypeptides are resolved (Okamura et al, 1974). These were originally assigned apparent molecular weights of 21, 24, and 28 kD, and these molecular weights are still often used to refer to the

different polypeptides. The apparent molecular weights found, however, depend strongly on the acrylamide concentration used, so the molecular weights 21, 24, and 28 kD are useful only if the acrylamide concentration in the gel is stated. Another nomenclature used ubiquitously is L, M, and H, standing for light, medium, and heavy, as revealed by mobility under SDS-PAGE. A serious problem with assigning molecular weights to RC polypeptides is the use of water-soluble standards. The RC polypeptides, particularly the L and M subunits, are hydrophobic. From the DNA sequences of the RC polypeptides (Williams et al, 1983 and Youvan et al, 1984b), the L and M polypeptides have molecular weights of approximately 31 and 34.5 kD respectively. The discrepancy between molecular weights deduced from the DNA and assigned by SDS-PAGE is typically attributed to a higher level of SDS bound by hydrophobic polypeptides. At least in the case of the M subunit, this is not the whole story, because the electrophoretic mobility changes upon ethanol precipitation (see Chap. III, part 3). The complete explanation of the "anomalous" migration of the L and M subunits on SDS-PAGE is still to be determined.

Recently the techniques of molecular genetics have been applied to structural questions concerning RC's. Williams et al (1983) used the known amino-terminal sequences of RC subunits from Rps. sphaeroides, strain R-26 (Sutton et al, 1982) to synthesize oligonucleotides complimentary to the genes for those polypeptides. The oligonucleotides were used as probes to locate the genes for H, M, and L. The sequence of M was determined and shown to be consistent with sequence data from internal peptides generated by

cleavage with trypsin and chymotrypsin. The sequence for the L subunit from Rps. sphaeroides is nearing completion (Williams et al, 1984). Youvan et al (1984a,b) used genetic data to localize the reaction center genes of Rps. capsulata to small regions of an R' plasmid constructed by Marrs (Marrs, 1981). The genetic approaches included marker rescue with known restriction fragments of the R' plasmid (Taylor et al, 1983), and transposon mutagenesis of the R' plasmid (Zsebo, 1984). The indicated regions of DNA were sequenced, yielding the sequences of all three RC subunits, as well as several intriguing unidentified open reading frames. These sequence data have been corroborated by amino-terminal sequences determined on isolated subunits (Chap. III, part 2) and by preliminary fragmentation data. No sequences from internal peptides are yet available for RC's from Rps. capsulata.

The functions of each of the RC polypeptides are not fully understood. It is possible to remove the H subunit, either electrophoretically (Okamura et al, 1974) or through the use of LiClO_4 and centrifugation on a sucrose gradient (Feher and Okamura, 1978). The particles obtained, referred to as LM, retain the absorption spectrum of RC's, indicating that the BChl's and BPhe's are retained in approximately their native environment. It is not known which pigments are bound to L and which are bound to M, or whether each pigment binding site contains domains contributed by both L and M. LM particles retain the ability to perform primary charge separation, although the kinetics of charge recombination are slower than in RC's (Debus et al, 1981). A slower back reaction suggests that the spatial relationship between the primary donor and

the quinone acceptor has been altered in the process of removing H. The ability to photobleach the primary donor in the LM complex indicates that only L and M are required to convert a photon into chemical potential.

The question remains as to the function of the H subunit. An extreme view would be to classify the H subunit as an intransigent impurity in RC preparations, an impurity that persists only because no one has devised the correct procedure to remove it without altering the properties of the LM complex. Whether H "belongs" in an RC preparation depends on the assembly of the three subunits within the chromatophore membrane. If the three subunits are always associated with each other in the membrane, in a 1:1:1 stoichiometry, then the H subunit should be considered a part of the RC. It should be noted that an association of H with LM in detergent extracts is not sufficient to say that they associate in the membrane. H has been shown to form chemical cross-links with L and M as well as with components of both light-harvesting complexes (Peters et al, 1983). These results indicate that within the membrane H is in close contact with L and M as well as the light-harvesting complexes. H may have a role in maintaining the lateral organization of complexes within the chromatophore membrane. Recently, a protein thought to be the H subunit, based on apparent molecular weight and reaction with anti-RC antibodies, was detected in Rps. sphaeroides cells devoid of intracytoplasmic membranes (Chory et al, 1984). (The presence of L and M could not be absolutely excluded due to their poor antigenicity.) These workers proposed a role for the H subunit as a site of insertion of BChl

and/or BChl-binding proteins into the developing chromatophore membrane. It remains to be seen whether H has any role in directly modulating the behavior of the protein-bound cofactors. It is this alteration of solution behavior which produces the extremely high quantum yield of photon capture that is at the heart of the catalysis of the photosynthetic reaction center.

In this thesis, I will describe work directed mainly at increasing our understanding of the protein portion of the RC. Only by knowing the structure of the protein, and by becoming proficient at performing the type of protein chemistry routinely applied to water-soluble enzymes, can we address the questions of interactions between the RC protein and the bound cofactors. As discussed above, these interactions between protein and cofactors may lie at the heart of the mechanism of energy conversion and stabilization. In Chap. II I will present a new isolation procedure for RC's. This procedure, utilizing affinity chromatography on equine cyt c, completely avoids the use of precipitating agents during the purification. In Chap. III I will describe the isolation of H, M, and L, using preparative electrophoresis, and report the amino-terminal sequences determined from the isolated subunits. I will describe fragmentation schemes developed for the three RC subunits. In Chap. IV I will describe experiments using quinone analogues as electron acceptors in RC's and PSII.

CHAPTER II: ISOLATION OF REACTION CENTERS USING AFFINITY CHROMATOGRAPHY

1. INTRODUCTION

In this chapter I will describe a new isolation procedure for bacterial reaction centers. After solubilization in detergent, RC's are bound to an affinity column bearing equine cyt c. From studies of electron donation to the RC (Ke et al, 1970 and Prince et al, 1974), equilibrium binding to the RC (Rosen et al, 1980 and Overfield and Wraight, 1980) and crystallographic analysis (Dickerson, 1980), equine cyt c is an excellent analogue to bacterial cyt c_2 . Affinity chromatography has been used successfully to isolate reaction centers from Rps. sphaeroides, strain R-26, and Rps. capsulata, strain KZR8A1. Both of these strains lack colored carotenoids. The absence of carotenoids is probably important only in that crt^- mutants typically lack some or all of the proteins of the light-harvesting II complex. Presumably, it is the altered organization of the chromatophore membrane, due to the altered LHII complex, which facilitates the solubilization of RC's from crt^- mutants. Affinity chromatography on equine cyt c has also been applied to obtain preparations highly enriched in RC's from wild-type Rps. sphaeroides. These preparations, however, were consistently contaminated by light-harvesting pigments and proteins.

The goals in developing a new isolation procedure were to obtain pure RC's, in high yield, and in as short a time as possible. The need to minimize the time of purification stems from the inevitable degradation of any protein until it is separated from the cellular proteases released upon rupture of the cell. Another goal of the purification was to avoid the use of precipitating agents during the

purification. This goal is based on a "hunch" that crystallization of RC's was impeded by using RC's prepared by ammonium sulfate precipitation. This hunch is consistent with, but hardly proved by, the observation that crystals of RC's from Rps. viridis were obtained from RC's isolated by HPLC, without the use of precipitants (Michel, 1982). Also, the crystals obtained with RC's from Rps. sphaeroides are of consistently higher quality if the RC's are prepared using the affinity column technique (G. Feher, personal communication).

The procedure used to isolate RC's has evolved over the last three years. Consequently, the procedure used to isolate RC's from R-26 is somewhat different from that used with KZR8A1. These differences primarily reflect the development of the procedure, and not species differences between sphaeroides and capsulata. A real species difference is the higher amount of a 44 kD protein found in R-26 as compared to a 42 kD protein found in KZR8A1. The procedure used with R-26 RC's will be described first, followed by the procedure used with KZR8A1.

2. Rps. sphaeroides, strain R-26

a. Method

Cells of Rps. sphaeroides, strain R-26, were grown photosynthetically in modified Hutner's medium as described by Austin (1976). After picking single colonies from aerobically grown yeast extract plates, all culturing was done anaerobically in a light chamber at 30-32 C. Illumination was from incandescent light bulbs, at an intensity of approximately 6 mW/cm². Three days after

inoculation, cultures were subcultured into larger flasks, using a 5% inoculant. Final cultures were grown in 9 l serum bottles. A cell density of 3 g/l culture was typically obtained in the large bottles. After harvesting, cells were washed once with 100 mM Na phosphate/10 mM EDTA, pH 7.5 (P/E buffer) and frozen at -70 C in P/E containing 10% glycerol.

The cytochrome c column was prepared as previously described (Godinot and Cautheron, 1979). Essentially, cyanogen bromide-activated Sepharose was treated with horse heart cyt c (Sigma, Type VI) at pH 8.0 for 90 min. After removal of uncoupled cyt c (typically less than 5% of added cyt), any residual cyanogen bromide sites were blocked with ethanolamine. By reacting the sites with this small amine, any reaction between the column and proteins (e.g. RC's) added later is avoided.

RC's were assayed by measuring the reversible photobleaching of the primary donor absorption band (865 nm in R-26, 850 nm in KZR8A1). Side-illumination of a Cary 14 sample compartment was achieved with a 600 W tungsten filament lamp, filtered through 5 cm of H₂O and a Baird Atomic B-3X filter (maximum transmission at 593 nm). The photomultiplier tube was protected from scattered actinic light by an interference filter (865 nm or 850 nm) or a Corning 7-59 filter. In either case, the combination of excitation and detection filters had an absorbance greater than 4.5 throughout the region 200-1000 nm. In some cases saturated bleaching signals were not obtained. In these instances the bleaching expected at saturating intensity was extrapolated from a double reciprocal plot of $1/\Delta A$ vs. $1/I$. Actinic light intensities were measured with a LYCOR LI-185 light meter.

Linear double-reciprocal plots were routinely observed.

Alternatively, photobleaching of RC's was measured in the IR2 mode of the Cary 14. For R-26 RC's, the measured difference extinction coefficient of $1.2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was used (Straley et al, 1973). The difference extinction coefficient has not been measured in Rps. capsulata, but it is probably similar to that in R-26. For the purpose of determining RC yields in a purification only relative amounts of protein need be known, and thus it is not necessary to know the difference extinction coefficient.

Chromatophores were prepared in the P/E buffer system (Fraker and Kaplan, 1971). This buffer prevents aggregation of the chromatophores during the isolation. Aggregation of chromatophores results in trapping of unwanted cellular fragments, including cytoplasmic membranes and ribosomes, in the final pellet.

To prepare chromatophores, cells were thawed, suspended to a concentration of 12 g/40 ml P/E, and broken by two passes through a French pressure cell at 20,000 psi. Unbroken cells and large membrane fragments were removed by centrifugation at 18,000 rpm (SS34 rotor), first for 15 min, then for 40 min. The supernatant from the second spin was diluted at least two fold with P/E and centrifuged at 50,000 rpm (60Ti rotor) for 50 min. The pellet was resuspended to the same volume as was originally loaded into the ultracentrifuge, and respun at 50,000 rpm for 50 min. During resuspension of all chromatophore pellets, care was taken to discard any grey material at the very bottom of the pellet. The resuspension and recentrifugation was repeated. The pellet was resuspended in 10 mM Hepes/ NaOH, pH 8.0 (Hepes buffer) to an absorbance of 5.0 cm^{-1} at 865 nm. (In the

next step chromatophores were resuspended to this same volume.) Enough LDAO was added from a 30% (w/v) solution to give a final detergent concentration of 0.05%. Chromatophores were centrifuged as above, and the pellet resuspended. Chromatophores were pelleted once more as above. This final pellet was designated "purified" chromatophores. Purified chromatophores were used directly for the RC isolation, or alternatively were stored at -20 C in a minimum volume of Hepes containing 50% glycerol. Chromatophores stored in glycerol were collected by dilution with Hepes buffer and centrifugation as above.

Fresh chromatophores, or chromatophores collected from glycerol, were resuspended in Hepes buffer to one tenth the volume required to give an absorbance of 5.0 in the above step. LDAO was added from a 30% stock solution to a final concentration of 1.0%. The suspension was centrifuged at 18,000 rpm (SS34 rotor) for 25 min. The supernatant was loaded onto a cyt c column preequilibrated with Hepes buffer containing 1.0% LDAO. The size of the column was such that the minimum cyt c/RC ratio was 10. The height of the column was typically 2-2.5 times the diameter.

When the solubilized chromatophores were completely loaded, the column was washed with 5 column volumes of Hepes/1% LDAO. The eluent buffer was switched to Hepes/0.1% LDAO/25 mM KCl. The column was washed with this buffer until the eluent had an absorbance at 280 $< 0.1 \text{ cm}^{-1}$. The RC's were eluted with Hepes/0.1% LDAO/100 mM KCl. At this point the peak fractions contained RC's which were approximately 80% pure, as judged by a ratio A_{280}/A_{800} of 1.5.

Further purification of the RC's was achieved by repeating the affinity chromatography. Fractions from the first column which had a

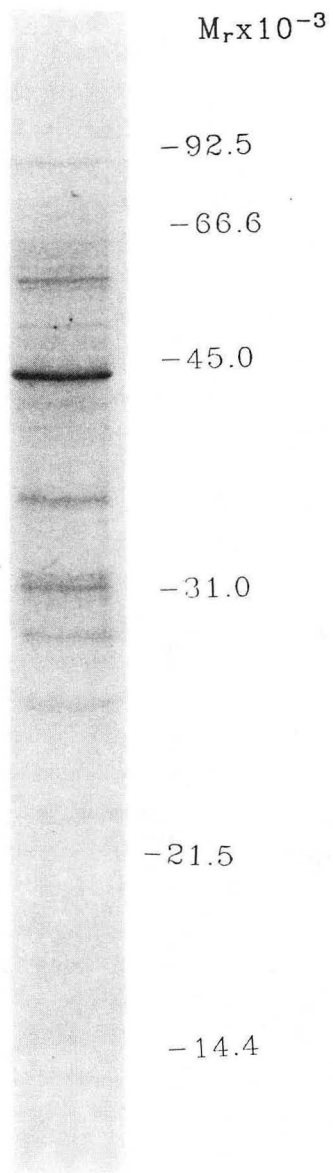
ratio $A_{280}/A_{800} < 1.70$ (approximately half the eluted RC's) were dialyzed overnight against Hepes/0.1% LDAO and reloaded onto a fresh cyt c column equilibrated with the same buffer. No protein was seen eluting from the column during the second loading. Reaction centers were immediately eluted in 100 mM KCl. All procedures after cell rupture were carried out at 4 C under a green safe light.

b. Results.

The cyt c column is capable of greatly enriching reaction centers from a very crude membrane preparation. The purest RC's are obtained, however, if non-RC proteins are removed to as great an extent as possible prior to loading. The purpose of washing the chromatophores with 0.05% LDAO is to remove proteins which are peripherally associated with the chromatophore membrane. It is particularly important to remove a protein of apparent molecular weight 44 kD. Fig. II-1 shows the polypeptides contained in the 0.05% LDAO extract of chromatophores. The most prominent band is the 44 kD protein. RC's can also be seen in the extract. The loss at this step is approximately 10% of the total RC, as measured by reversible bleaching at 865 nm. This loss is an unavoidable consequence of removing the 44 kD protein. Other faint bands can also be seen in the extract. At this point their identity is unknown.

The chromatophores obtained after extraction with 0.05% LDAO are shown in Fig. II-2. The 44 kD protein is still the most prominent band in the chromatophores. The RC bands H, M, and L are the next most prominent bands. Attempts to use a higher LDAO/BChl ratio in the first LDAO extraction step, to remove more of the 44 kD protein,

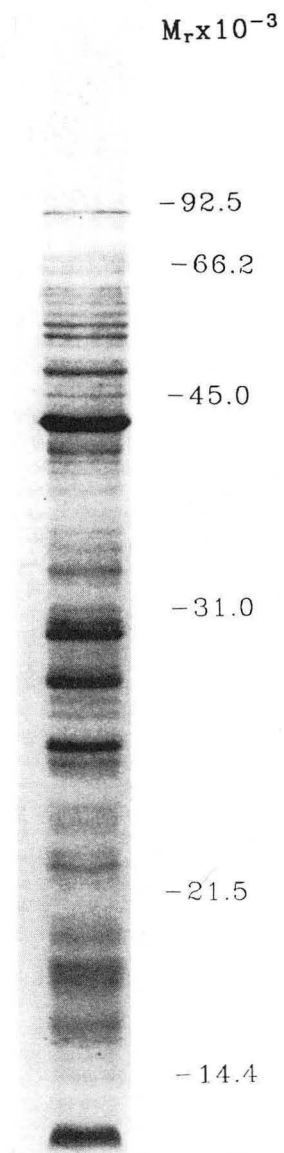
Fig. II-1. SDS-PAGE (13% acrylamide) of proteins found in the supernatant when chromatophores from R-26 are extracted with 0.05% LDAO.



XBB 847-5635

Fig. II-1

Fig. II-2. SDS-PAGE (13% acrylamide) of proteins in chromatophores from R-26 after LDAO extraction.



XBB 847-5633

Fig. II-2

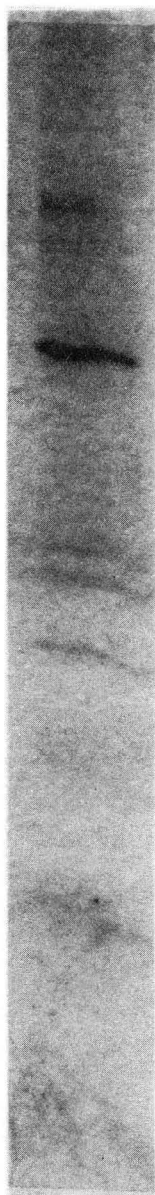
resulted in substantial loss of RC's into the supernatant.

The fractions eluted from the column during loading and 1% LDAO washing contain LH proteins as well as some RC subunits (data not shown). The material eluted during washing with 25 mM KCl is shown in Fig. II-3. These fractions consist primarily of the 44 kD protein, as well as traces of RC subunits.

The peak RC fractions from the first cyt c column have a ratio A_{280}/A_{800} of 1.5. Using a value of 1.2 for this ratio for "pure" RC's (Feher and Okamura, 1978), the purity of these peak fractions is estimated to be 80%. To continue with the purification, fractions with $A_{280}/A_{800} < 1.7$ were pooled. These pooled fractions (average purity = 70%) represent approximately half the RC's eluted from the first column. After RC's were eluted from the second column, fractions with $A_{280}/A_{800} < 1.3$ were pooled and treated as purified RC's. The ratio of 1.3 corresponds to a purity of approximately 90%. Typically, 30-35% of the RC's present in the original chromatophores was contained in the final purified RC fraction.

The proteins present in the final RC fraction which are detectable by SDS-PAGE are shown in Fig. II-4. In addition to the prominent RC bands, at least three other bands are visible. The 44 kD protein is visible, as is a protein migrating directly behind the H subunit. The protein directly above H is probably the cyt c_1 protein of the bc_1 complex. The evidence for this will be discussed in the section on capsulata RC's. Finally, a protein with an apparent molecular weight of 49 kD is faintly visible. In the original description of this procedure (Brudvig et al, 1983), the 49 kD protein was attributed to a LM aggregate. This is consistent with assignments

Fig. II-3. SDS-PAGE (13% acrylamide) of proteins which eluted from the cyt c column during a wash with 0.1% LDAO, 25 mM NaCl. (Chromatophores from Rps. sphaeroides, strain R-26).



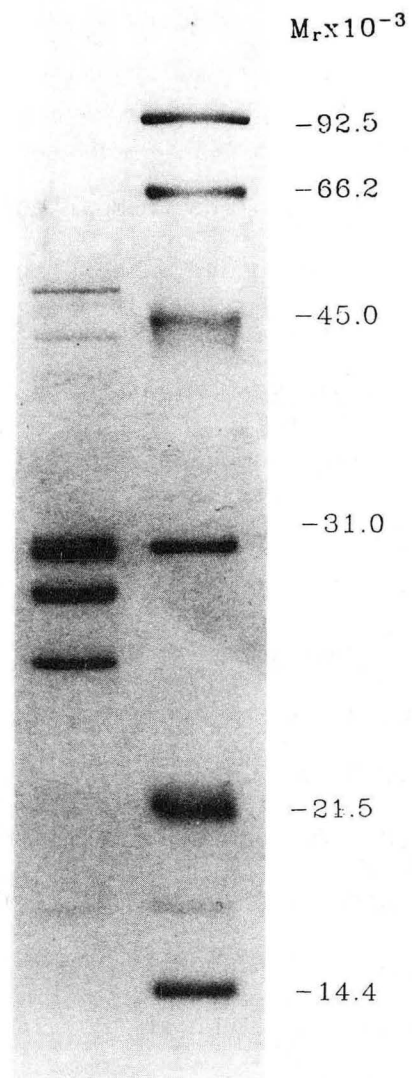
$M_r = 44,000$

H
M
L

XBB 849-6683

Fig. II-3

Fig. II-4. SDS-PAGE (13% acrylamide) of reaction centers from R-26 isolated by cyt c affinity chromatography. Left, reaction center proteins. Right, molecular weight standards.



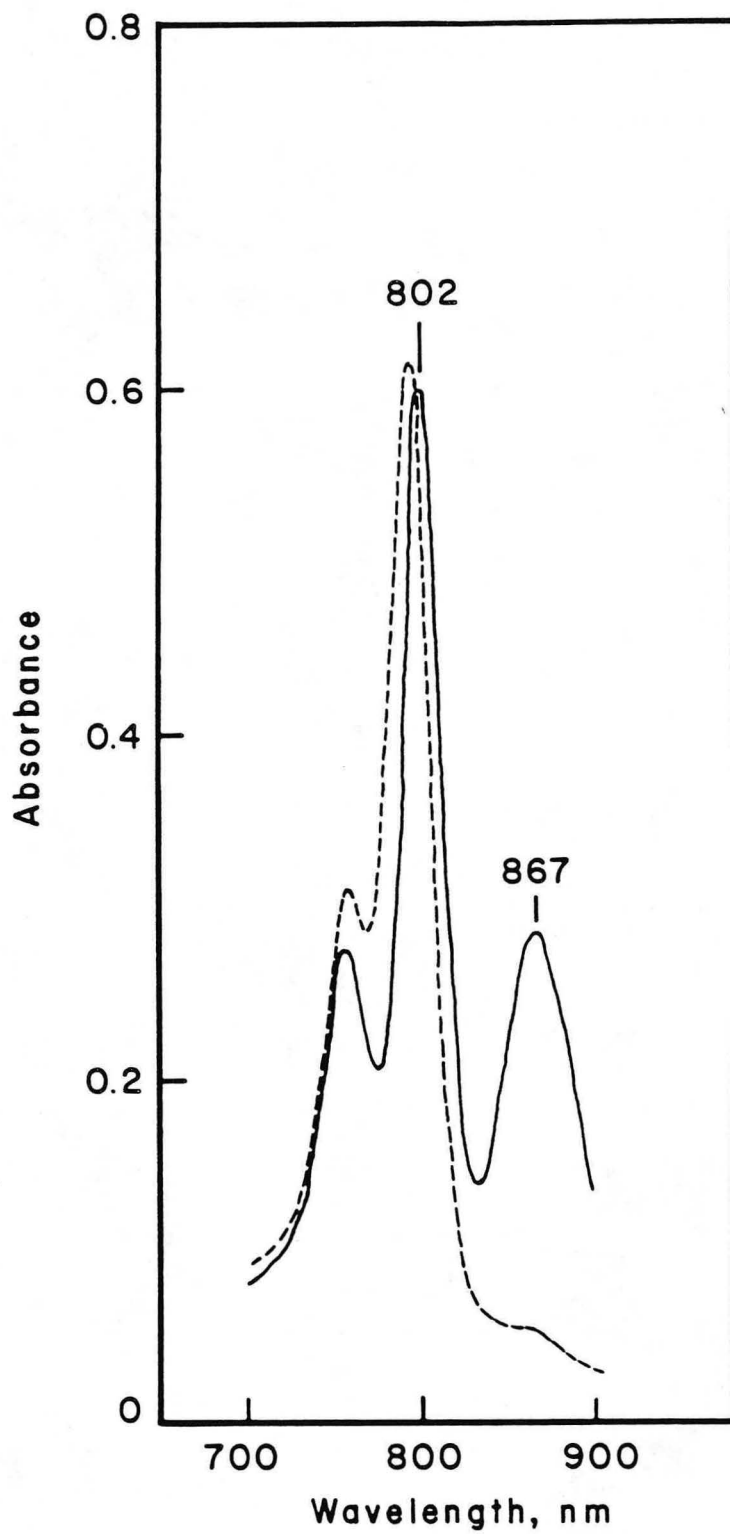
XBB 847-5634

Fig. II-4

from other laboratories in which material with a similar electrophoretic mobility was seen in RC preps (Thorner et al, 1980; Okamura et al, 1974). I now believe this assignment to be incorrect. Preparations of RC's from KZR8A1 do not show material at this position on the gel (see Fig. II-10). Furthermore, when elution from the cyt c column is achieved with a continuous salt gradient, rather than a step gradient, material is seen at 49 kD which elutes prior to the RC fractions (see Fig. II-7). This suggests that the material is unrelated to RC's.

The near-IR absorption spectrum of purified RC's from R-26 is shown in Fig. II-5. The spectrum shows three maxima in the near-IR, characteristic of bacterial RC's. The relative intensities of the three peaks are very similar to results seen previously (Feher, 1971; Clayton and Wang, 1971; Okamura et al, 1974). The only pronounced difference is that in the RC's prepared on a cyt c column, the 765 maximum is slightly lower (5-10%) relative to the 865 peak. Both protein-bound BPhe and free BChl absorb in this region, so it cannot be determined from the absorption spectrum whether the altered ratio is due to a loss of BPhe in this purification, or due to a slight contamination by free BChl in other purifications. The ratio of the three IR maxima indicates the absence of significant contamination by light-harvesting BChl. Not shown in Fig. II-5 are the maxima at 535-540 nm, 365 nm (with a shoulder at 380), and at 280 nm. These peaks are indistinguishable in shape and relative intensity from the peaks seen by other workers. The positions of the maxima are very similar to those in other reports. This suggests that interactions between the various pigments, or between the pigments and the protein, are not

Fig. II-5. Near-IR absorption spectrum of reaction centers from R-26 isolated by cyt c affinity chromatography. Solid trace is spectrum obtained under weak illumination. Dotted trace is bleached spectrum obtained under strong (10 mW/cm^2) illumination.



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Fig. II-5

disrupted by the solubilization and isolation. It is possible that all solubilizations result in exactly the same disruptions, thus giving the same absorption spectrum. This seems unlikely, as my own observations suggest that once degradation begins, the absorption spectrum does not have reproducible features, but rather is different for each isolation.

Fig. II-5 also shows the bleached spectrum of RC's from R-26 (dotted trace) obtained in the IR2 mode of the Cary 14. The bleached spectrum is the same as published spectra (Feher, 1971; Okamura et al, 1974) and indicates that the activity of the RC is preserved during isolation.

Fig. II-6 shows a light-induced EPR signal observed from RC's at 10 K. This signal, centered at $g=2.003$, has a peak-peak linewidth of 9.8 Gauss, the same as seen previously (Feher et al, 1975). This peak is due to the oxidized primary donor and has the g -value expected for $BChl^+$. The line-width of the signal (narrower than that of $BChl^+$ in solution by a factor of 1.4) has been interpreted in terms of a $BChl$ dimer model for the primary donor. See Chapter I for reservations about this interpretation. The $g=2.003$ signal in Fig. II-6 decayed with a $1/e$ time of 19 ms at 8 K (kinetic trace not shown). This decay time is the same as seen before, both in isolated RC's and in whole cells (McElroy et al, 1974). The preservation of this decay time from whole cells to isolated RC's suggests that the spatial and orientational relationships between the primary donor and primary acceptor are not altered during isolation. The broad EPR signal associated with the reduced quinone acceptor, magnetically coupled to the Fe(II), was not visible in this RC preparation. This is most

Fig. II-6. Light-induced EPR signal of reaction centers from R-26 isolated by cyt c affinity chromatography.

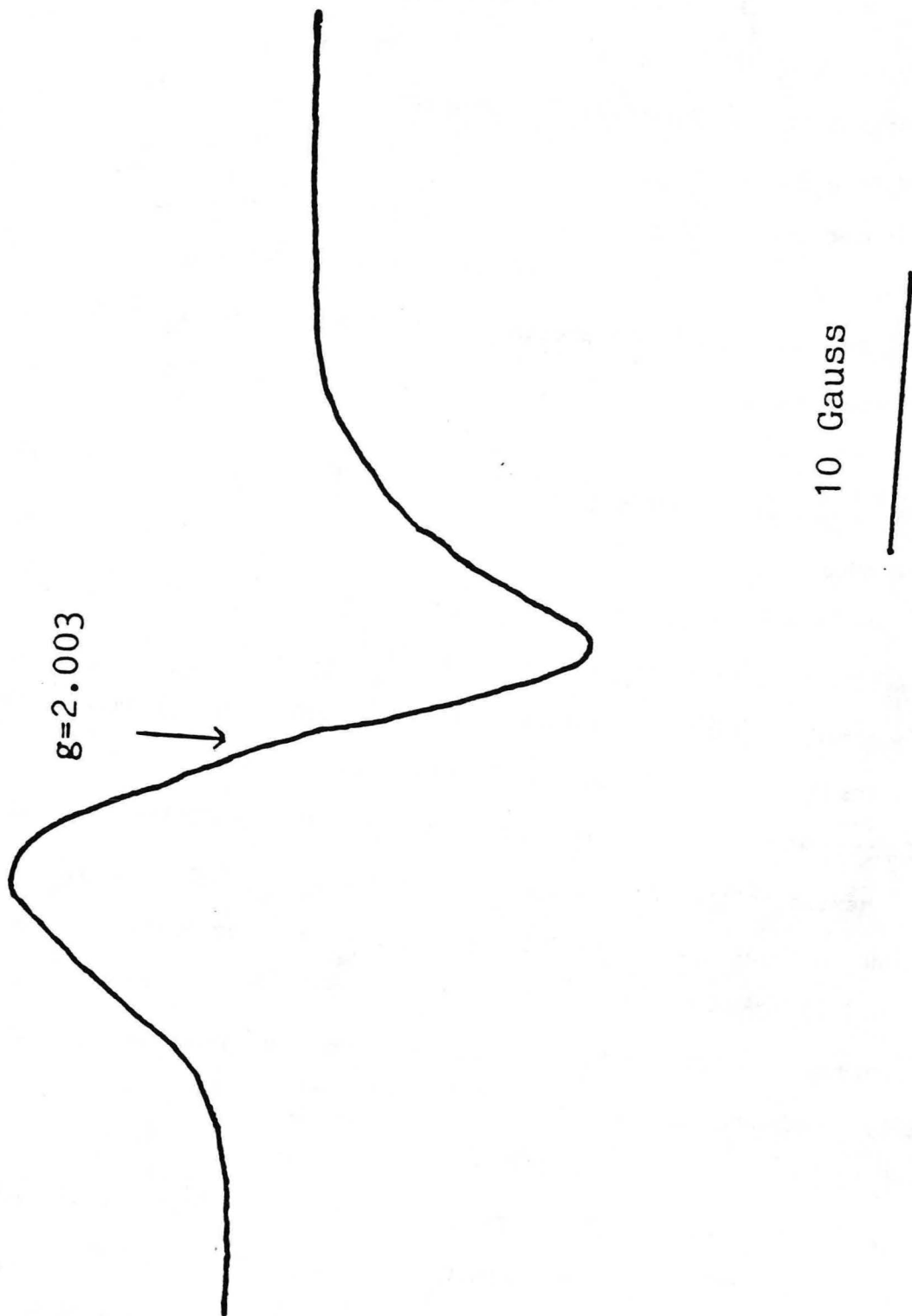


Fig. II-6

likely due to the conditions of observation. To detect this signal, Feher et al used a 10x more concentrated RC sample, and observed the EPR signal at 2 K (Feher and Okamura, 1978, p. 366). At the time these measurements were made, we were unable to attain such a low a temperature within the EPR cavity. It should be noted that no atomic absorption measurements were made to confirm the presence of iron in this RC preparation. The presence of iron is only suggested by its presence in many other preparations of RC's, using various protocols.

3. Rps. capsulata, strain KZR8A1

a. Method

RC's were purified from Rps. capsulata, strain KZR8A1. This strain was chosen because of its similarities to R-26. KZR8A1, generated by transposon mutagenesis (Zsebo and Hearst, 1984), contains a transposon insert in the carotenoid E gene, a gene involved in the biosynthesis of carotenoids (Scolnik et al, 1980). KZR8A1 fails to synthesize colored carotenoids and lacks the 14 kD protein typically found associated with the B800-850 light-harvesting complex of Rps. capsulata (Cogdell and Thornber, 1980). The near-IR absorption spectrum of KZR8A1 is characteristic of mutants containing only RC and B870 complexes (Bolt et al, 1982).

Stock cultures of KZR8A1 were stored at -70 C in 10% glycerol. The stocks originated from single colonies picked from a yeast extract plate inoculated from the original isolate of KZR8A1. The single colonies were grown aerobically in liquid peptone/yeast extract (PYE) for 12 hr, mixed with an equal volume of PYE containing 30% glycerol and frozen in 1 ml aliquots. To grow large quantities of cells, a 1 ml

aliquot was thawed and added to 20 ml PYE. Growth was continued aerobically for 24 hrs. This culture was used to inoculate 500 ml PYE, and aerobic growth was continued for 12 hrs. The 500 ml culture was used to inoculate a 9 l bottle containing RCV medium. After 8-10 hr in the dark, the 9 l bottles were placed in the light chamber and grown for 3 days. Illumination and growth temperature were as described for R-26 cells. Between 3 and 4 g cells/liter of medium were obtained in the 9 l bottles. Harvesting and washing of cells was identical to procedures used for R-26 cells.

Aerobic growth of the inoculant cultures was chosen for two reasons. First, the doubling time under aerobic conditions is 3-4 times less than the doubling time under photosynthetic growth conditions. Second, the chances of enriching a revertant mutant containing a complete B800-850 complex are decreased by minimizing the time of photosynthetic growth.

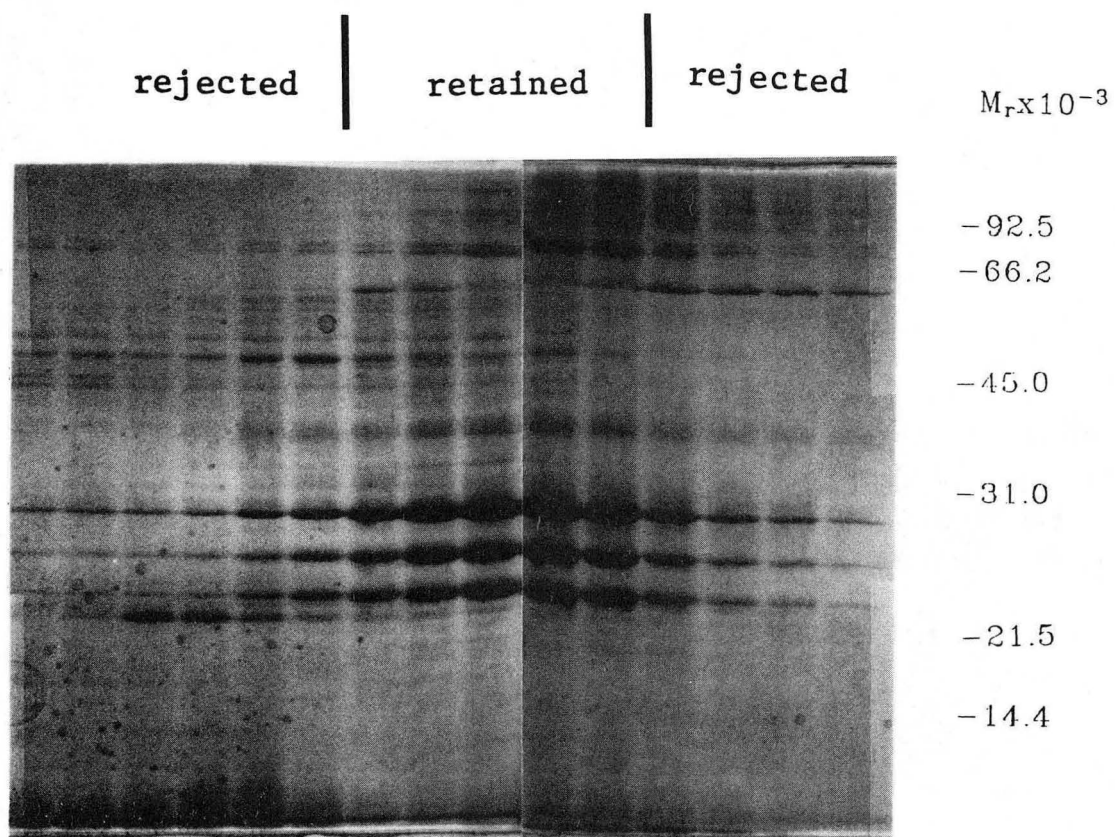
Chromatophores from KZR8A1 were prepared as described for R-26 cells except that 10 mM Tris/HCl, pH 8.2 at 4 C (Tris buffer) replaced Hepes buffer. For the LDAO wash, the absorbance at 870 nm was set to 10.0 cm^{-1} (instead of 5.0 at 865 nm). Preparation of the cyt c column was also as in part 2.

Chromatophores were resuspended to one fifth the volume originally required to give an absorbance of 10 cm^{-1} , and solubilized with 0.6% LDAO. A lower LDAO concentration was used (compare to 1.0% for R-26) in the belief that the position of the absorbance band due to the primary donor was shifted to shorter wavelenghts upon solubilization. I no longer believe this to be the case, as is discussed in the results section. Unsolubilized material was removed

by centrifugation at 48,000xg for 40 min. The supernatant was loaded onto a cyt c column (mol cyt c/mol RC =10) preequilibrated with Tris buffer containing 0.6% LDAO. After loading, the column was washed with the pre-equilibration buffer until the absorption at 760 nm was less than 0.1. After lowering the LDAO concentration to 0.05%, a non-linear salt gradient was passed over the column. The non-linear gradient was formed from two solutions of Tris buffer/ 0.05% LDAO: one solution containing no salt, the other containing 300 mM NaCl. The non-linear gradient was formed in a BRL gradient maker (model 750). The volume of the solution containing no salt was twice the volume of the solution containing 300 mM NaCl. The height of each solution in the gradient maker was made equal by inserting plexiglass rods into the 300 mM NaCl solution. The purpose of the non-linear gradient is to increase the resolution obtained prior to RC elution, but still to minimize the volume of the fractions containing RC's. The continually increasing gradient in salt concentration minimizes trailing of the RC fractions. The volume of the gradient was approximately 15x the volume of the cyt c column.

The ratio of A_{280}/A_{800} is not a straightforward indicator of purity in RC's from KZR8A1 (see below for a possible explanation). Therefore, the fractions from the first cyt c column which were to be used in further purification steps were selected on the basis of SDS gels. Gels were examined visually, and only fractions in which RC polypeptides were the most prominent bands seen on the gel were used. Fig. II-7 shows an example of how these selections were made. Fractions are shown in the order eluted from the cyt c column. The lanes marked "retained" contain fractions which were pooled and used in the next

Fig. II-7. SDS-PAGE (14% acrylamide) of proteins contained in fractions from the first cyt c column (chromatophores from Rps. capsulata, strain KZR8A1). Fractions are shown left to right in the order eluted from the cyt c column. Fractions labelled retained were pooled, dialyzed, and applied to a second cyt c column to obtain pure reaction centers.



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Fig. II-7

step of the purification. The fractions eluting prior to the retained fractions were rejected because of the proteins with apparent molecular weights of 22 kD and 49 kD. Fractions eluting after the retained fractions were rejected because of the protein with apparent molecular weight of 62 kD.

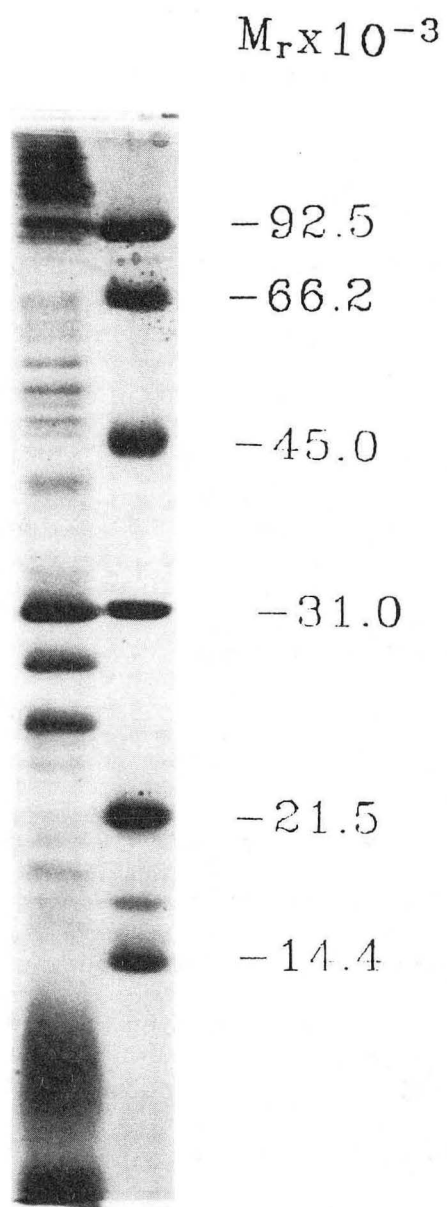
Pooled fractions were dialyzed and loaded onto a new cyt c column. Immediately after loading, elution was begun with a salt gradient identical to the gradient used on the first column. The peak fractions obtained from this second column appear to be pure by SDS gels (see Fig. II-10).

b..Results

As with strain R-26, the best results are obtained by washing the chromatophores prior to solubilization. Proteins present in KZR8A1 chromatophores after washing with 0.05% LDAO are shown in Fig. II-8. The protein at 42 kD is much less pronounced than the 44 kD protein in R-26. These protein may be related, because both stain for heme with tetramethylbenzidine (Zsebo, 1984; Worland, unpublished observations). The lower levels of the 42 kD protein in KZR8A1 is a distinct, reproducible difference between this strain and R-26. It is an important difference for using cyt c affinity chromatography, because the 44 kD protein was the major contaminant in RC's from R-26.

When RC's from KZR8A1 were eluted with a step gradient, or a linear continuous gradient, significant contamination was seen by a protein with an apparent molecular weight of 30-31 kD. The composition of these impure RC's was investigated by re-loading the material on a cyt c column, and eluting with a non-linear gradient

Fig. II-8. SDS-PAGE (14% acrylamide) of proteins in chromatophores from KZR8A1 after extraction with 0.05% LDAO. Left, chromatophore proteins. Right, molecular weight standards.



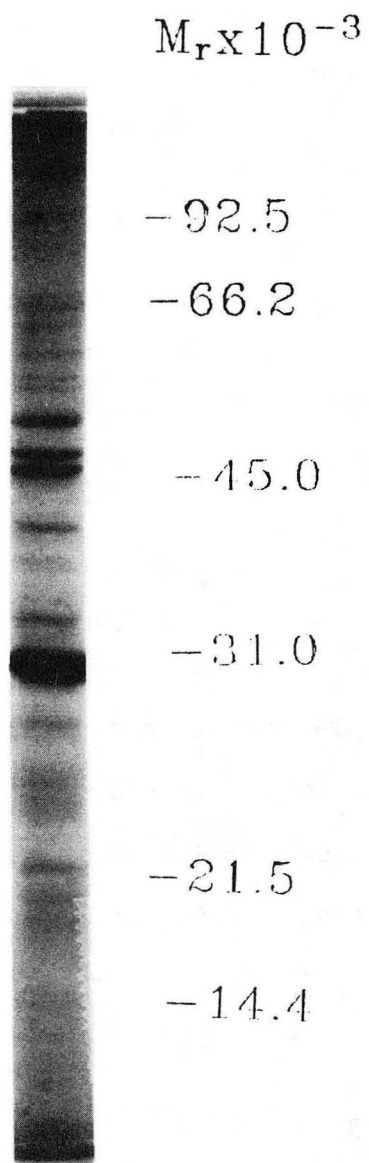
XBB 847-5664

Fig. II-8

as described in the Methods section. Fig. II-9 shows the material which eluted prior to the RC fractions. The heavily stained band has an apparent MW of 30 kD. These fractions were concentrated further and loaded onto a preparative gel (see Chap. III for techniques of preparative electrophoresis). Upon completion of the preparative gel, a blood-red band was visible to the eye, suggesting the presence of a covalently bound heme group. This red band corresponds to the 30 kD band in Fig. II-9, as shown by rerunning a portion of the removed slice from the preparative gel on an analytical gel. Several pieces of evidence suggest that this protein is the c_1 portion of the bc_1 complex. The cyt c_1 of the bc_1 complex has an apparent molecular weight of 30-31 kD on SDS gels (Yu et al, 1984). The c_1 portion of the bc_1 complex donates electrons to cyt c_2 (the analogue to the cyt c used in preparing the affinity column), and thus might be expected to bind to equine cyt c, as do RC's. The complete bc_1 complex must recognize equine cyt c, because the complex can be purified on a equine cyt c column (Trachiotis and Worland, unpublished observations). Finally, the heme groups of all c-type cytochromes are covalently attached to the protein, so it is expected that the heme group would be retained during preparative electrophoresis. Based on these observations, the band at 30-31 kD, seen eluting prior to RC's when a continuous, nonlinear salt gradient was used (KZR8A1 preparation) and seen contaminating RC's when a step gradient was used (R-26 preparation, Fig. II-4), is tentatively identified as the cyt c_1 of the bc_1 complex.

Fig. II-10 shows a gel of the RC's obtained after a second cyt c column. The three RC polypeptides are the only readily visible bands.

Fig. II-9. SDS-PAGE of proteins which eluted from a cyt c column prior to reaction centers when a non-linear salt gradient was used (chromatophores from KZR8A1). The heavily-stained band migrating just below the $M_r=31,000$ standard corresponds to a visibly red band.



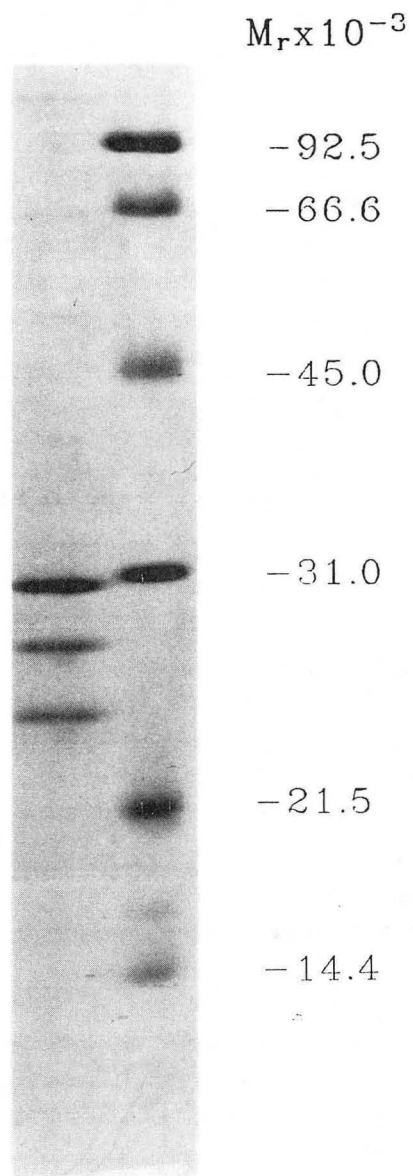
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Fig. II-9

On the original gel very faint bands are visible with apparent molecular weights of 50 kD, 65 kD, and >100 kD. An average yield of 25% (3 purifications) was obtained for RC's of comparable purity to those shown in Fig. II-10.

The absorption spectrum of purified RC's is shown in Fig. II-11. This spectrum is similar to that of RC's from R-26, with two exceptions. The position of the longest wavelength maximum is at 850 nm (865 nm in R-26), and the ratio of A_{280}/A_{800} is 1.95 instead of 1.25. The longest wavelength maximum has been seen at 850 nm before in RC's from the A1a⁺ mutant of Rps. capsulata (Nieth et al, 1974). These workers reported that a light-minus dark spectrum of untreated chromatophores from A1a⁺ showed a maximum bleaching at 865 nm, but the difference spectrum was not shown. I have observed that membranes and RC's from carotenoidless mutants of Rps. capsulata are very susceptible to irreversible bleaching. In chromatophores, this irreversible bleaching has a maximum at 870 nm and shows no zero-crossing at 800 nm, indicating destruction of the B870 light-harvesting complex. A true indication of the RC bleaching spectrum in untreated chromatophores can only be obtained by subtracting the irreversible bleaching from the total bleaching. The reversible component of the light-dark difference spectrum for chromatophores from KZR8A1 is shown in Fig. II-12. The maximum bleaching is seen at 852 nm. Also visible is a zero-crossing at 800 nm, as seen in R-26 RC's. From this spectrum I conclude that, in RC's isolated from KZR8A1, detergent solubilization has not altered the position of the absorption maximum attributed to the primary donor. The position of this peak is also at 850 nm in RC's isolated from A1a⁺

Fig. II-10. SDS-PAGE (12.5 % acrylamide) of reaction center proteins from KZR8A1 isolated by cyt c affinity chromatography. Left, reaction center proteins. Right, molecular weight standards.



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Fig. II-10

Fig. II-11. Absorption spectrum of reaction centers from KZR8A1 isolated by cyt c affinity chromatography.

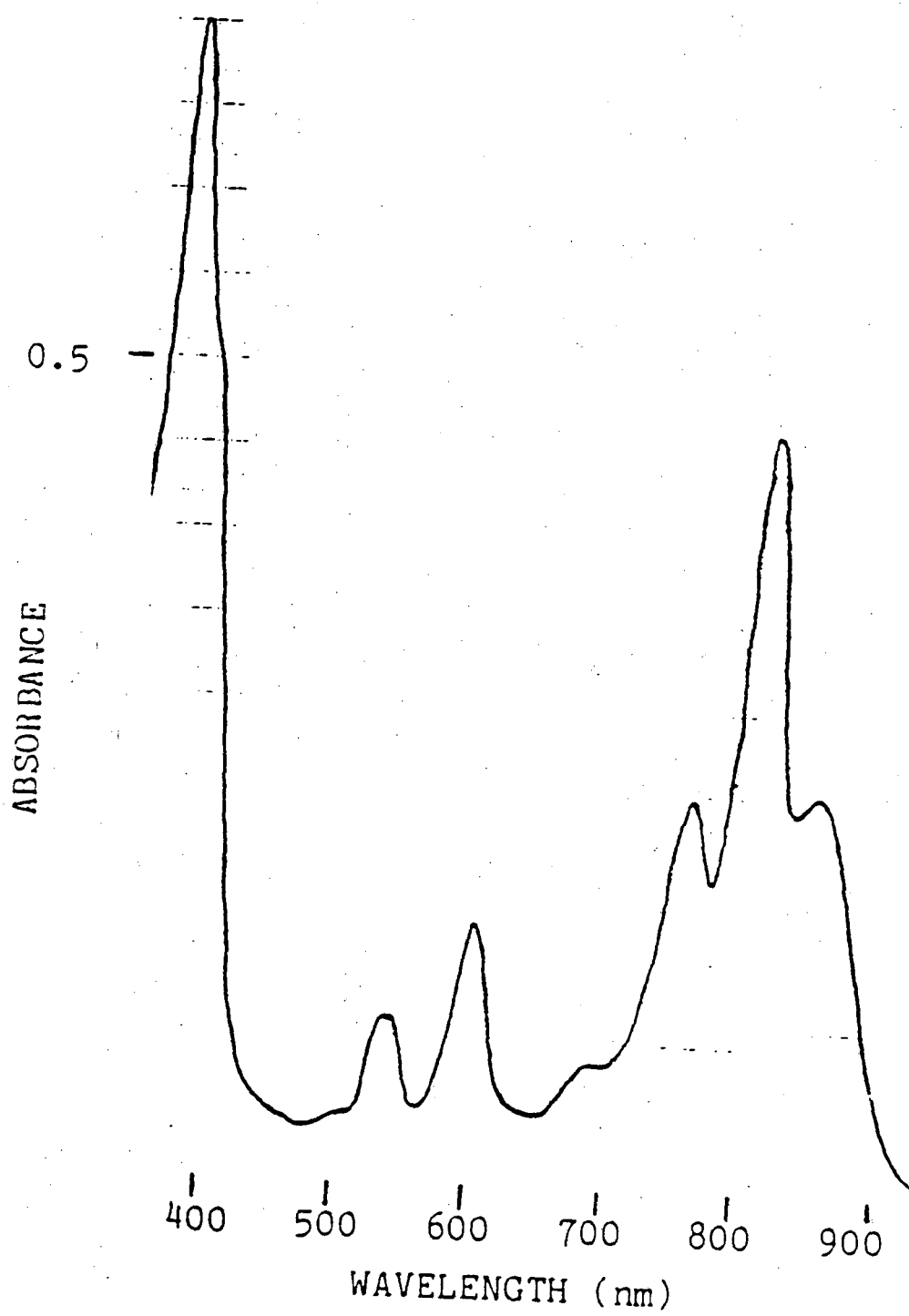


Fig. II-11

Fig. II-12. Reversible component of a light-minus-dark spectrum of chromatophores from KZR8A1. An irreversible bleaching, centered at 870 nm, was subtracted from the light-minus-dark spectrum.

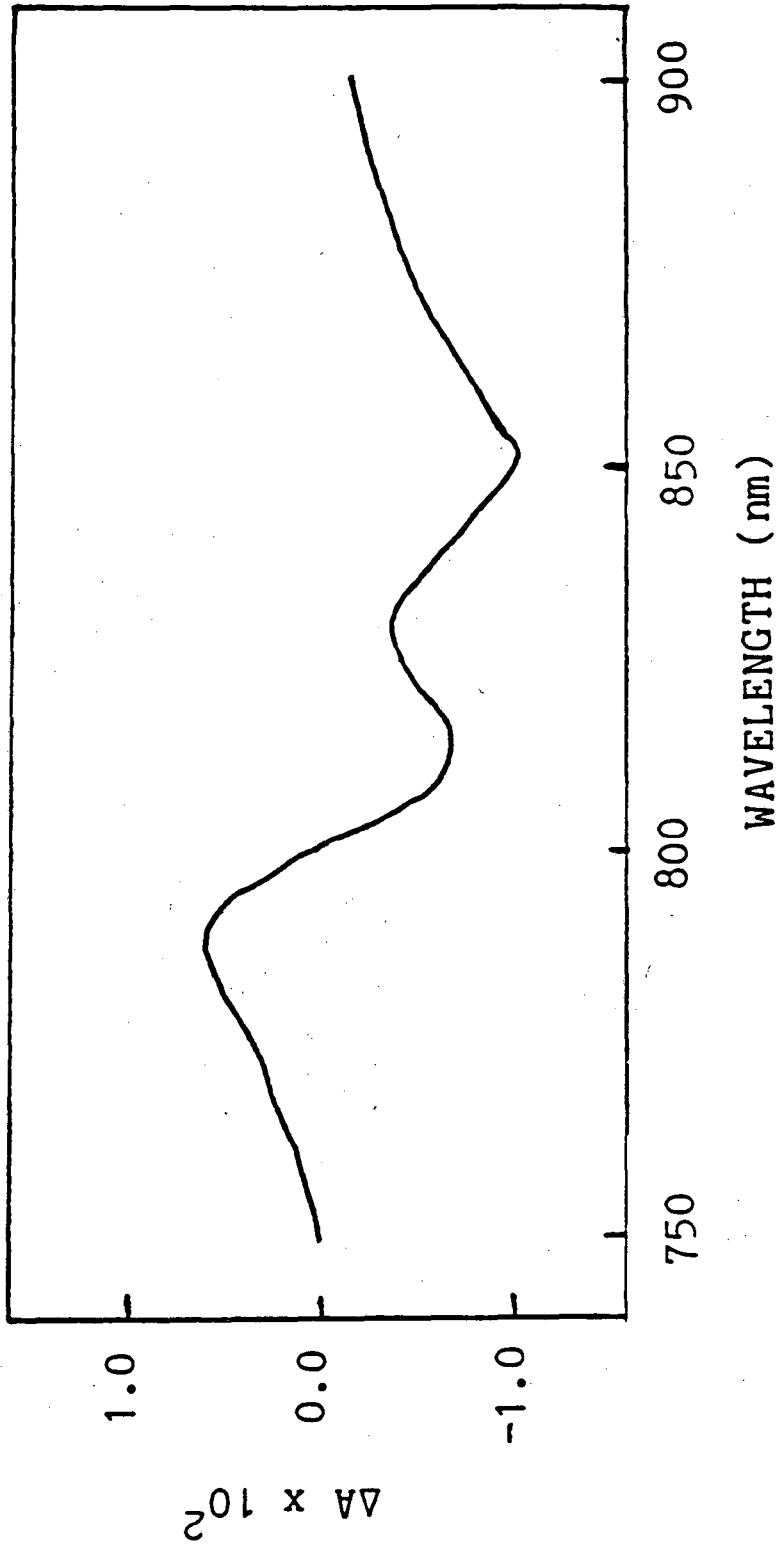


Fig. II-12

(Nieth et al, 1974) and in RC's isolated from BW604, another carotenoidless mutant of Rps. capsulata (Tabbutt, unpublished observations). Thus it appears that all carotenoidless mutants of Rps. capsulata, and perhaps the wild type strain, have RC's with an IR maximum at 850 nm.

Except for the position of the band at 850 nm, and the relative intensity of the band at 280 nm, the spectrum in Fig. II-11 is very similar to the spectrum of RC's from R-26. The spectrum differs somewhat from that seen by Nieth et al (1974). Relative to the absorption at 800 nm, I see a stronger absorption at 850 nm and a weaker absorption at 760 nm, as compared to Nieth et al. The decreased absorption at 850 nm in their spectrum may represent loss of absorption due to the primary donor. The increased absorption at 760 nm seen in their preparation could be due to free BChl or protein bound pigment which has been converted from BChl to BPhe. Based on the fact that relative intensities of the maxima are similar in Fig. II-11 and Fig. II-5, I conclude that Fig. II-11 represents the correct spectrum for RC's from carotenoidless mutants of Rps. capsulata.

In my hands, attempts to measure a fully bleached spectrum of RC's from KZR8A1 always resulted in irreversible bleaching at 870 nm. Consequently, fully reversible bleachings were measured at several light intensities. The reversible bleaching appeared to vary hyperbolically with light intensity, as shown by linear double reciprocal plots ($1/\Delta A$ vs. $1/I$). A double reciprocal plot was used to estimate the bleaching which would be obtained if a saturating intensity were used. A typical double reciprocal plot is shown in Fig. II-13. The points are all colinear, suggesting that an analysis

assuming simple saturation behavior is justified. The extrapolated maximum bleaching observed in such a double reciprocal plot was always between 85 and 90% of the total absorption at 850 nm (data from 4 independent isolations of RC's from KZR8A1). In RC's from R-26 the fully bleached signal corresponds to 86% of the absorption at 865 nm (Fig. II-5, and Feher, 1971). Therefore, the RC's isolated from KZR8A1 by affinity chromatography contain a fully oxidizable primary donor.

The ratio A_{280}/A_{800} in Fig. II-11 is 1.95 (the lowest ratio I have seen for RC's from KZR8A1 is 1.89). For pure RC's from R-26 this ratio is 1.20 (Feher and Okamura, 1978). There are at least three possible explanations for the increased absorption at 280 nm relative to 800 nm. One, the RC preparation may contain non-RC proteins. The gel in Fig. II-10 clearly rules out this explanation. Two, the RC's may have lost absorption at 800 nm, due to loss of BChl. This is unlikely, in light of the fact that the relative intensities of all maxima except that at 280 nm are similar to those in R-26. If the high A_{280}/A_{800} is due to loss of BChl, it must be the case that all BChl and BPhe species are lost to the same extent, while the binding constant to cyt c is unaltered. This is an unlikely explanation. Third, the absorption at 280 nm may be due to another chromophore in addition to the aromatic amino acids in the RC protein. A chromophore absorbing in the UV was extracted from KZR8A1 RC's during the process of removing quinones (see chap. IV for the complete procedure for quinone extraction.) Briefly, RC's at a concentration of 1.5 μ M were extracted with 0.3% LDAO and 0.2 mM o-phenanthroline. The extracted RC's were retarded on a cyt c column, and the extracted material

Fig. II-13. Double-reciprical plot showing light intensity dependence of absorbance changes at 850 nm in reaction centers from KZR8A1. Bleaching extrapolated to infinite intensity by linear least-squares regression. Absorbance of reaction center sample at 850 nm = 0.023.

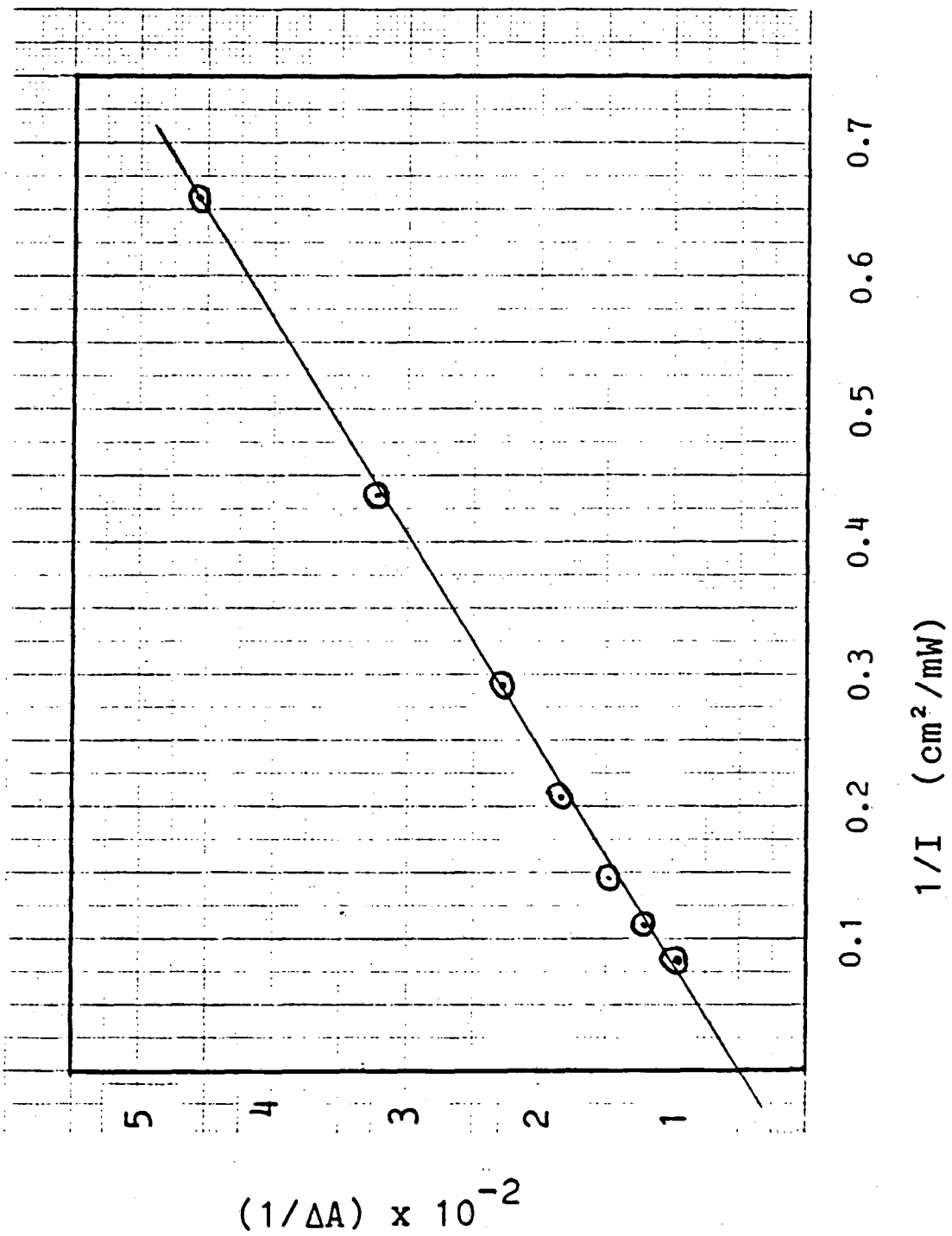


Fig. II-13

recovered in the eluent.

Fig. II-14 shows a spectrum in the UV of material recovered from an o-phenanthroline/LDAO extraction of RC's from KZR8A1. Absorption maxima are visible at 264 nm and 228 nm, with shoulders at 324, 308 and 288 nm. When 100 ul of this material, having an absorption at 265 of 10 cm^{-1} , was loaded onto an SDS gel, no material stainable by Coomassie blue was seen. This suggests that the material is not protein. Also, the absorption maximum is not as expected for protein. Neither is the spectrum consistent with the material being a nucleic acid. At this point no assignment can be made as to the nature of the material shown in Fig. II-14. Because KZR8A1 contains a transposon in a carotenoid biosynthetic gene, it is interesting to speculate about a carotenoid precursor giving rise to a spectrum like that in Fig. II-14. In plants, precursors to visible carotenoids contain 3 conjugated double bonds (Porter and Spurgeon, 1979). In organic solvents, all-trans hexatriene has absorption maxima at 268, 258, and 247 nm. The extinction coefficients for these maxima are between 30,000 and 40,000 $\text{M}^{-1} \text{ cm}^{-1}$. Clearly, the spectrum in Fig. II-14 is not that of hexatriene, but may have contributions from conjugated double bonds. If an extinction coefficient of 35,000 is postulated for the material shown in Fig. II-14, a ratio of A_{280}/A_{800} of 1.95 for pure RC's from KZR8A1 would suggest two molecules of this unknown species per RC molecule. This is not unreasonable for a carotenoid or carotenoid precursor.

Clearly, the discussion concerning carotenoid precursors is purely speculative. It is provided merely to suggest possible future experiments. The extraction of the material shown in Fig. II-14 does,

however, provide an explanation for the ratio of $A_{280}/A_{800}=1.95$ which is seen for RC's containing no non-RC proteins.

Fig. II-14. UV spectrum of material extracted from reaction centers from KZR8A1.

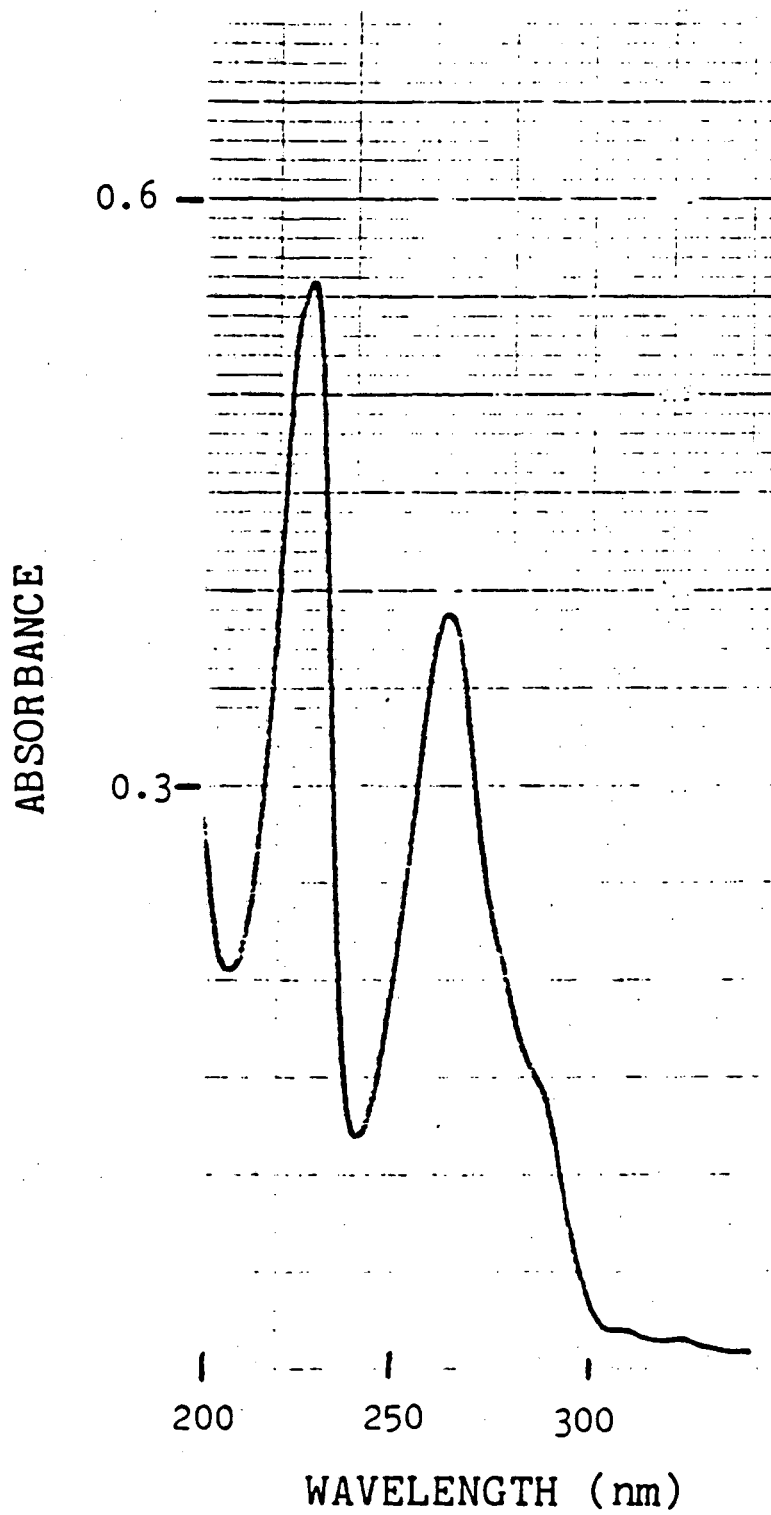


Fig. II-14

CHAPTER III: ISOLATION AND CHARACTERIZATION OF REACTION CENTER POLYPEPTIDES

1. INTRODUCTION

Reaction centers from Rps. capsulata and Rps. sphaeroides contain three polypeptide subunits (see Chaps. I and II). At least two of these subunits, referred to as M and L, interact with the cofactors involved in the primary photochemistry catalyzed by the RC. The third subunit, H, has no known function in binding cofactors. To understand the interactions between protein and cofactors one must know the amino acid sequence of each subunit. The sequences of the RC subunits from Rps. capsulata have been deduced from the DNA sequences of the corresponding genes (Youvan et al, 1984a,b). Sequence data determined directly from the polypeptides complement the data available from DNA sequences. Amino-terminal sequence data determined from the protein also allows one to assess the extent of post-translational processing at the amino-terminus of the polypeptides. This information is not available from the DNA sequences alone.

A complete understanding of protein-cofactor interactions, and of the importance of these interactions for catalysis, requires knowledge of which regions of the protein interact with which cofactors. One approach to this problem is the determination of the protein structure by X-ray crystallography. This has been difficult with hydrophobic proteins such as the RC, but recently significant progress has been made (Michel, 1982 and Allen and Feher, 1984). An alternate approach to the problem is to use reactive cofactor analogues to form covalent attachments between the protein and the cofactor analogues. Separation of the subunits then permits assignments as to which cofactors

interact with which subunits. The binding site of the tightly bound quinone (see Chap. I) in bacterial RC's was assigned to the M subunit using an azido derivative of anthraquinone (Marinetti et al, 1979). Covalent attachment of a cofactor analogue to a particular subunit, followed by fragmentation of the polypeptide chain, allows one to assign the binding region for that cofactor to a small region of one subunit. With this information in hand one can begin to address the question of the role of protein-cofactor interactions in the function of the protein.

In this chapter I will describe experiments which laid the ground work for mapping cofactor binding sites to specific regions of the RC protein. Preparative gel electrophoresis was used to isolate the three RC subunits from strain KZR8A1. Samples of each subunit were submitted for determination of the amino-terminal sequences using a gas-phase sequenator. Comparison of the sequences determined from the protein and the sequences deduced from the DNA confirms the DNA sequences and establishes the extent of processing at the amino-terminus of each subunit of RC's from strain KZR8A1. Finally, fragmentation procedures were developed for the RC subunits dissolved in aqueous SDS.

2. ISOLATION AND AMINO-TERMINAL SEQUENCES OF REACTION CENTER POLYPEPTIDES

a. Methods

The source of RC'S for these studies was Rps. capsulata, strain KZR8A1. This strain, and the growth protocols used, are described in Chap. II. Isolation of chromatophores was as described in Chap. II, up to the LDAO wash step. At this point, SDS was substituted for LDAO, as described below. This is possible because active RC's were not desired.

Chromatophores were depleted of non-BChl proteins by resuspending to an absorbance of 10 at 870 nm, adding SDS to 0.05% final concentration and centrifuging at 250,000 x g (60Ti rotor, Beckman) for 1 h. The resulting pellet was resuspended in a minimum volume of 10 mM Tris-HCl (pH 8.0) and applied to the preparative gel after solubilization (see below). Alternatively, the resuspended pellet was combined with an equal volume of glycerol and stored at -20 C. Storage for at least 6 months had no effect on electrophoretic separation of the RC subunits.

Preparative electrophoresis was performed as described (Hunkapiller et al, 1983) using the Tris/glycine buffer system (Laemmli, 1970). Chromatophores containing 250 nmol BChl, as estimated by absorbance at 375 nm (Neufang et al, 1982), were loaded onto a slab gel which was 3 mm thick and 14 cm wide. The stacking gel, containing 6 % acrylamide, was 2 cm long. The separating gel, containing 12.2 % acrylamide, was 11 cm long. The chromatophore suspension was suitably diluted with an application buffer to yield the following final concentrations: SDS, 75 mM; dithiothreitol, 65 mM; Tris-HCl (pH 6.8), 65 mM; glycerol, 10%. Bromophenol blue was used as the tracking dye. After addition of the application buffer, samples were maintained at room temperature for 30 min prior to loading. Samples were not heated. Stacking gels were run at 20 mA until the bromophenol blue was just entering the separating gel. Separating gels were run at 25 W constant power, using 4 l of anode buffer as a thermal reservoir. Electrophoresis was continued for 20 min after the bromophenol blue exited the gel, for a total time in the separating gel of approximately 200 min.

Gels were stained for 3 min in isopropanol/acetic acid/water (35/10/55, v/v) containing 0.5% Coomassie blue. Gels were destained for 5-7 min in methanol/acetic acid/water (50/10/40, v/v) with several changes of destaining solution. The three reaction center bands, clearly visible against a faint background, were cut out by hand using a sharp razor blade. Each gel slice retained was approximately 3 mm wide. Slices of the gel (4-5 mm) between H and M and between M and L were discarded. Gel slices were washed and proteins electroeluted at 4 C in ammonium bicarbonate as described (Hunkapiller et al, 1983). After elution each sample was lyophilized, resuspended in a minimum volume of water, and treated with 9 volumes of absolute ethanol at -20 C. After 48 h at -20 C the precipitated protein was recovered by centrifugation. At this point the proteins would not dissolve in water. H and M were redissolved in 0.1% SDS. L was redissolved in 0.1 % SDS, 50 mM dithiothreitol (DTT). The requirement for DTT to redissolve L is probably related to the 5 cysteine residues in this subunit. (H contains no cysteine residues, while M contains only one.) The resuspended proteins were frozen until applied to the sequenator.

Isolated subunits were submitted to Dr. K. Wilson, Cetus Corp., for sequencing. Amino-terminal sequences were determined on a gas-phase sequenator (Applied Biosystems, Foster City, CA). Approximately 10 ug of protein was applied per run. Phenylthiohydantoins were resolved by HPLC (Hunkapiller and Hood, 1983) on a Cyano column (IBM Instruments) and assigned (by K. Wilson) without prior knowledge of the DNA sequence.

b. Results and Discussion

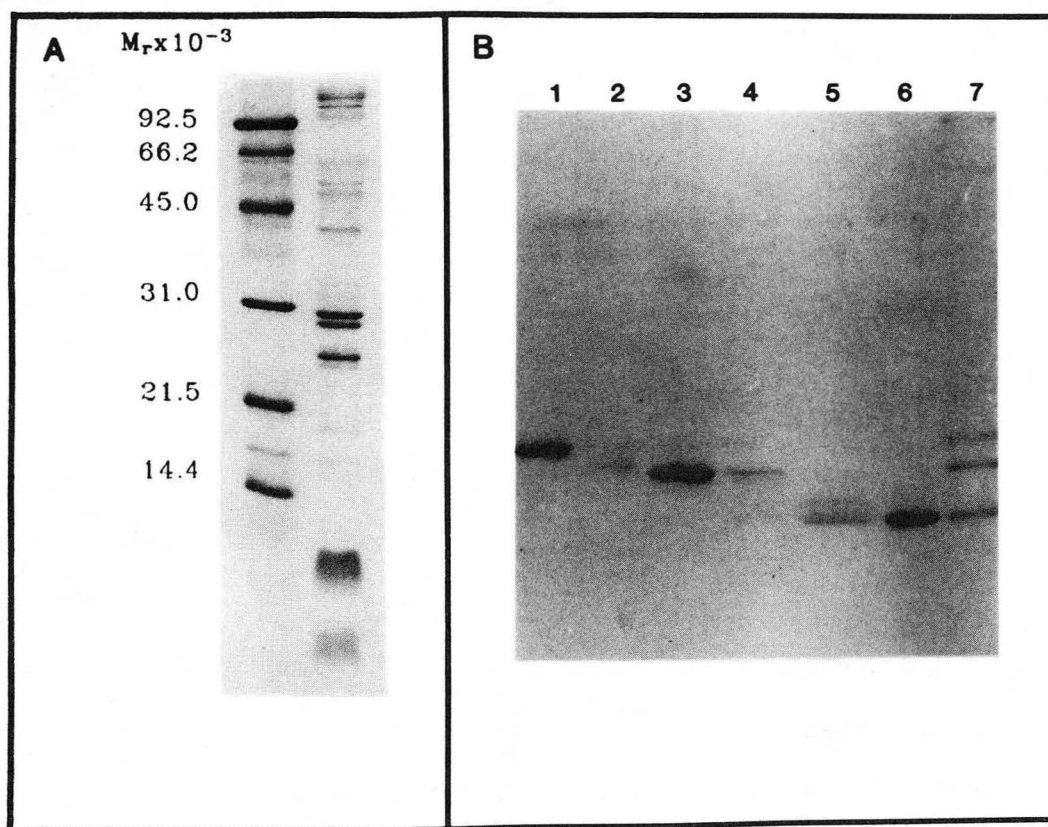
Fig. III-1A shows an electrophoretogram of chromatophore proteins remaining after extraction with 0.05% SDS. The three reaction center subunits are the most prominent bands. Proteins of the light-harvesting complexes are visible below the $M_r=14,000$ standard. Acrylamide gradient gels resolve at least three light-harvesting proteins in this region (Zsebo, 1984). The presence of material responsible for the faint bands visible near the reaction center subunits did not prevent sequencing material prepared electrophoretically.

An electrophoretogram of the isolated subunits, after electroelution but prior to ethanol precipitation, is shown in Fig. III-1B. No cross contamination between the three subunits is visible. Heavily loaded gels reveal another protein in the M fraction, which nearly comigrates with M on the preparative gel. The discarded regions of the gel are also shown. The material loaded into the "discarded" lanes corresponds to 4 times as much of the preparative gel as was loaded into the lanes containing retained subunits. It is clear that the amount of material lost due to this precautionary step is small. Separation equivalent to that shown in Fig. III-1B was obtained with loads up to 1 mg of reaction center. This corresponds to approximately 350 ug of each subunit per 3 mm gel.

Several precautions must be taken if proteins are to be successfully sequenced after electrophoresis (see Hunkapiller et al, 1982a). The main difficulty is blockage of the amino-terminus, from reactions with components within the gel. Radicals remaining from the polymerization of the gel were minimized by letting the gels

Fig. III-1. SDS-PAGE of chromatophore proteins and isolated reaction center subunits from Rps. capsulata, strain KZR8A1.

A: (16% acrylamide) Left, molecular weight standards. Right, proteins remaining in chromatophores after extraction with 0.05% SDS. B: (12% acrylamide) reaction center subunits isolated by preparative SDS-PAGE. Lane 1, H subunit; lane 2, discarded material which migrated between H and M; lane 3, M subunit; lane 4, discarded material which migrated directly below the M subunit; lane 5, discarded material which migrated directly above the L subunit; lane 6, L subunit; lane 7 sample loaded onto the preparative gel (chromatophores after extraction with 0.05% SDS). The amount of material loaded into the "discarded" lanes corresponds to four times as much of the preparative gel as was loaded into the lanes containing retained subunits.



XBB 846-4242

Fig. III-1

stand at least 12 h after the initiation of polymerization, and by adding thioglycolic acid to the cathode buffer. Thioglycolic acid travels at the dye front and scavenges radicals still remaining in the gel. Certain unknown contaminants of even the highest grade commercial SDS seem to prevent the sequencing of proteins isolated using SDS gels (Hunkapiller et al, 1983). Consequently, SDS (Bio-Rad) was recrystallized twice from ethanol/water, according to the procedures described.

The amino-terminal sequences determined for the subunits after recovery from the gel are shown in Fig. III-2. Residual Coomassie blue remaining from the staining procedure prevented an accurate assignment of the amino acid released during the first cycle of the Edman degradation. The protein sequences deduced from the DNA sequences (Youvan et al, 1984a,b) for each subunit are shown for comparison. Exact agreement is seen between the residues assigned by Edman degradation and those deduced from the DNA sequences.

The amino-terminal sequences of H, M, and L have been determined by Edman degradation in Rps. sphaeroides strain R-26 (Sutton et al, 1982), following separation of the subunits by a large scale procedure. Likewise, the M and L subunits of Rhodospirillum (R.) rubrum G-9 have been isolated in large quantities and sequenced (Theiler et al, 1983). The isolation procedure described here does not yield such large quantities as do the other procedures, but has the advantage of being applicable to any set of proteins which can be resolved by electrophoresis. The ability to separate H, M, and L, all of which migrate within a 1.5 cm section of the gel, demonstrates the very high resolution of the preparative gels.

Fig. III-2. Amino-terminal sequences for the reaction center subunits from KZR8A1. A) sequences determined by automated Edman degradation on polypeptides eluted from preparative SDS gels. B) sequences deduced from DNA (Youvan et al, 1984a). Agreement between the two sequence determinations is indicated by a dash.

M Subunit

A) NH₂X Glu Tyr Gln Asn Phe Phe Asn Gln Val Gln Val Ala Gly Ala Pro Glu
 B) NH₂Met Ala -

L Subunit

A) NH₂X Leu Leu Ser Phe Glu Arg Lys Tyr Arg Val
 B) NH₂Met Ala - - - - - - - - - - - - - - - - -

H Subunit

A) NH₂X Val Gly Val Asn Phe Phe Gly
 B) NH₂Met - - - - - - - - - - -

The amino-terminal amino acid of each mature subunit can be identified by comparing the protein and DNA sequences (see Fig. II-2). For example, in the M subunit, the second residue in the protein (the first assigned residue) is the third residue deduced from the DNA. Thus the first amino acid in the mature M subunit is alanine and the amino-terminal methionine has been cleaved. Likewise, the methionine has been cleaved from the L subunit, leaving alanine as the first amino acid in the mature subunit. In the H subunit, the second residue in the protein is also the second residue deduced from the DNA sequence. Thus the amino-terminal methionine is retained in the H subunit. In no case is the processing of the amino-terminus of the RC subunits more extensive than removal of one amino acid.

The significance of the difference in processing for the M and L subunits versus the H subunit is not known. It is interesting to note other differences between these subunits. In Rps. capsulata, the genes for the M and L subunits are adjacent to each other and follow directly the genes for the B870 antenna complex, while the gene for the H subunit is 20,000 basepairs away (Youvan et al, 1984a). The M and L subunits bind all of the known pigments and quinones, whereas the H subunit is not known to bind any cofactors. (Okamura et al, 1982). The complex of L, M, and the bound cofactors is capable of undergoing reversible, light-induced charge separation. Hydropathy plots based on the deduced amino acid sequences suggest that L and M are very hydrophobic proteins which span the intracytoplasmic membrane several times, whereas H is much less hydrophobic and probably contains only one membrane-spanning segment (Youvan et al, 1984b). Recently, a protein thought to be the H subunit, based on apparent

molecular weight and cross reactivity to anti-reaction center antibodies, has been detected in cells of Rps. sphaeroides which were completely devoid of intracytoplasmic membranes (Chory et al, 1984). (The presence of M or L could not be absolutely excluded, because of their poor antigenicity.) These workers speculated on a role for the H subunit as a site of insertion of BChl and/or BChl-binding proteins into the chromatophore membrane. From the experiments concerning cofactor binding it is clear that the role of the H subunit is quite different from the role of the M and L subunits. The location of H on a different operon from M and L suggests a different history for H prior to insertion into the membrane. It remains to be seen whether the different processing of the reaction center polypeptides is related to their different histories prior to assembly or to their different roles once inserted into the chromatophore membrane.

The gene for the M subunit from Rps. sphaeroides has been sequenced (Williams et al, 1983). The amino-terminal sequences of all three RC subunits have also been determined (Sutton et al, 1982). The situation with respect to processing of the M subunit is the same as in Rps. capsulata. The amino-terminal methionine is cleaved, leaving alanine as the first amino acid in the mature protein. The first amino acid in the mature L subunit from Rps. sphaeroides is alanine; the first residue in the mature H subunit is methionine. Thus it appears that the processing of L and H are similar in Rps. capsulata and Rps. sphaeroides, but sequences for the L and H genes in Rps. sphaeroides are needed to confirm this.

Sequence homologies among reaction centers from Rps. capsulata, Rps. sphaeroides, and R. rubrum have been presented previously

(Youvan et al, 1984a and Theiler et al, 1983). For the sake of comparison, these homologies are presented in Fig. III-3. Of the 16 residues assigned here in the M subunit, 7 are conserved among all three organisms, 3 are conserved between Rps. capsulata and Rps. sphaeroides, 2 different residues are conserved between Rps. capsulata and R. rubrum, and 4 are not conserved between Rps. capsulata and either organism. All 10 of the residues assigned here in the L subunit are conserved among the three organisms. In the H subunit 5 of the 7 residues determined here are conserved between Rps. capsulata and Rps. sphaeroides. (The sequence for the H subunit from R. rubrum is not yet available.) The number of residues assigned here is not sufficient to draw conclusions concerning the functional significance of the homologies observed.

3. FRAGMENTATION OF REACTION CENTER POLYPEPTIDES

a. Introduction

Fragmentation procedures were developed for the RC subunits. The techniques used were adapted from procedures previously applied to water-soluble proteins. The major modification for fragmenting the RC subunits was the use of SDS as both solubilizing agent and denaturant. The fragmentation patterns were developed with full access to the deduced polypeptide sequences (Youvan et al, 1984b). The strategy involved looking for possible cleavage sites in the deduced polypeptide sequence. Particular attention was paid to those sites which would either provide a useful confirmation of the DNA sequence (by virtue of being towards the carboxy terminus of the protein) or allow isolation of potentially interesting fragments.

Fig. III-3. Sequence homologies in the amino-terminal regions for reaction center subunits from Rps. capsulata, strain KZR8A1 (this work), Rps. sphaeroides, strain R-26 (Sutton et al, 1982), and R. rubrum, strain G-9⁺ (Theiler et al, 1983). Homologous residues are indicated by *.

M Subunit

- A) NH₂Ala-Glu-Tyr-Gln-Asn-Phe-Phe-Asn-Gln-Val-Gln-Val-Ala-Gly-Ala-Pro-Glu-
- B) NH₂ * - * - * - * - * -Ile- * -Ser- * - * - * - * -Arg- * -Pro-Ala-Asp-
- C) NH₂Ser- * - * - * - * -Ile-Leu-Thr-Gly- * - * - * -Arg-Thr- * - * -His-

L Subunit

- A) NH₂Ala-Leu-Leu-Ser-Phe-Glu-Arg-Lys-Tyr-Arg-Val-
- B) NH₂ * - * - * - * - * - * - * - * - * - * - * - * -
- C) NH₂ * - * - * - * - * - * - * - * - * - * - * - * -

H Subunit

- A) NH₂Met-Val-Gly-Val-Asn-Phe-Phe-Gly-
- B) NH₂ * - * - * - * -Thr-Ala- * - * -

The peptidyl bond in the dipeptide Asp-Pro is more susceptible to acid cleavage than any other peptidyl bond found in a typical protein. This has been used (Landon, 1977) to fragment proteins specifically at Asp-Pro linkages, using limited acid hydrolysis. The technique has been extended to cleave at Asp-X sites, by elevating the temperature and decreasing the time of exposure to acid (Inglis, 1982). The deduced sequence of the H subunit contains an Asp at residue #149 and a Pro at residue #150. The deduced sequence for the M subunit contains an Asp at residue #80, and a Pro at residue #81. In both sequences, only one Asp-Pro peptidyl bond is found. Both of these Asp-Pro linkages were cleaved by slightly modifying the procedures of Landon.

Staphylococcus aureus V8 protease is an extracellular protease which cleaves after Glu and Asp residues (Drapeau et al, 1972). The specificity can be further restricted to cleavage only after Glu, by performing the cleavage in ammonium bicarbonate buffer (Houmard and Drapeau, 1972). The kinetics of cleavage at sites where Glu is followed by an amino acid with a bulky aliphatic side chain are so slow that these sites are essentially not cleavable. This further restricts the specificity of the Staphylococcus V8 protease. The activity of the V8 protease is unaffected by 0.2% SDS (Drapeau, 1978). This feature of the enzyme has been exploited in peptide mapping of proteins in slices from SDS gels (Cleveland et al, 1977). The protease activity is also unaffected by disulfide reducing agents such as DTT, presumably because the amino acid sequence is devoid of cysteines (Drapeau, 1978). Proteins can be denatured in 0.2% SDS, 100 mM DTT and subsequently cleaved with the V8 enzyme.

The deduced sequence for the L subunit contains 5 Glu's. Since one of these is followed by Val and another by Ile, only 3 sites of possible cleavage by S. aureus V8 enzyme are predicted. One "cleavable" Glu is the sixth amino acid from the amino-terminus. Cleavage at this site would be very difficult to detect on SDS gels, so only two sites of cleavage in the L subunit which would be detectable by SDS-PAGE are predicted. Cleavage was detected at one, and perhaps both, of these sites.

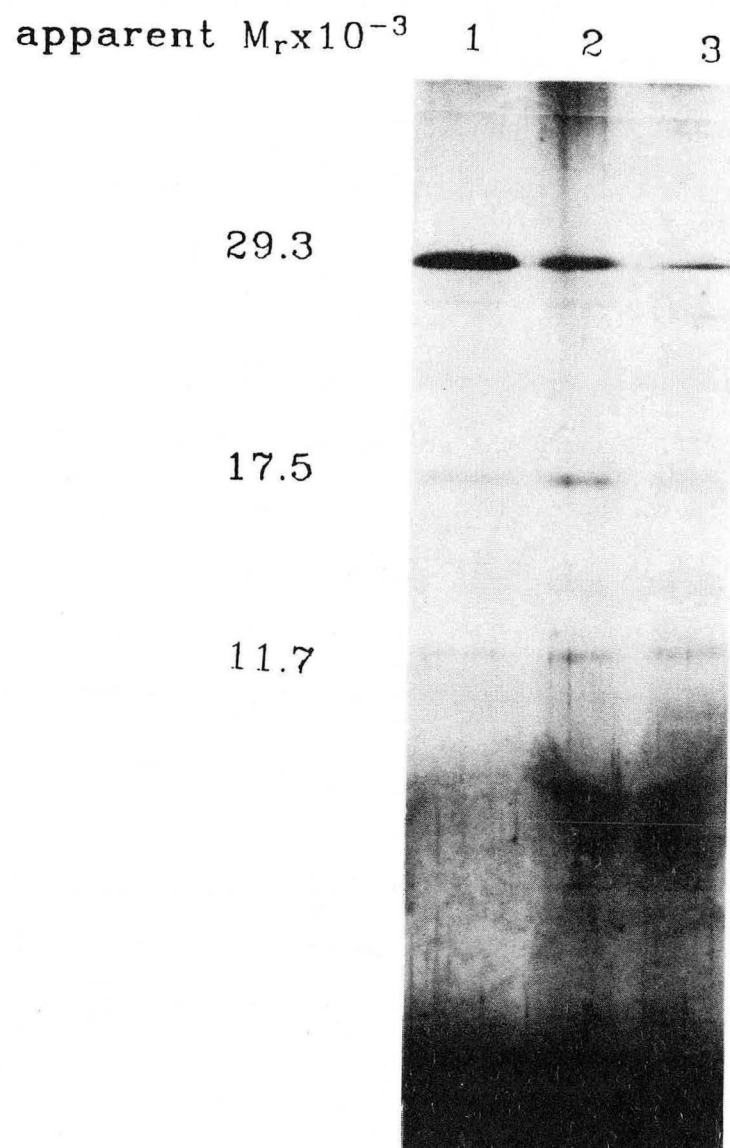
To perform the fragmentation studies, all three subunits were isolated electrophoretically, using the procedures described above. After ethanol precipitation, H and M were dissolved in 0.1% SDS. L was dissolved in 0.1% SDS, 100 mM DTT. All pellets were resuspended to a concentration of approximately 2-5 mg/ml, based on staining intensity with Coomassie blue and the maximum possible yield (100%) from the electrophoretic preparation procedure. All resuspended pellets were frozen at -20 C.

b. H Subunit

The resuspended H subunit was thawed and SDS was added to a final concentration of 2%. After 30 min at room temperature, 9 volumes of formic acid (purris grade, Fluka) were added and the solution incubated at 35 C. After acid cleavage, the sample was diluted 20 fold, frozen and lyophilized. The dried material was redissolved in 0.1% SDS (volume equal to 20x the original volume during acid cleavage) and relyophilized. Samples must be diluted prior to lyophilization because of the low freezing point of formic acid.

Fig. III-4 shows the time course of cleavage of the H subunit in

Fig. III-4. Time course of acid-cleavage of reaction center subunit H from KZR8A1. The H subunit was treated with 90% formic acid at 35 C. Aliquots were removed, lyophilized, and analyzed by SDS-PAGE (17% acrylamide). Lane 1, 19 h acid treatment; lane 2, 29 h acid treatment; lane 3, 40 h acid treatment.



XBB 847-5639

Fig. III-4

90% formic acid at 35 C. After 19 h two fragments can be seen in addition to the intact H subunit. At longer times, other fragments can be seen. The additional fragments are especially noticeable after 40 h of acid treatment.

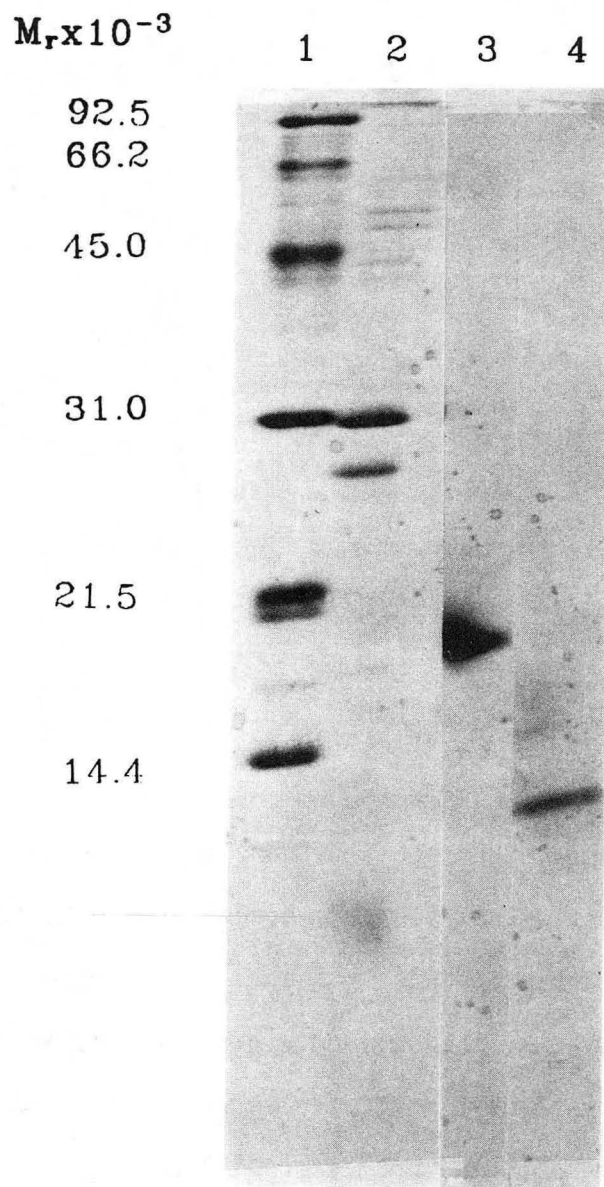
Cleavage on a preparative scale was performed for 24 h. After two lyophilizations, the contents of the cleavage reaction were separated on a preparative SDS gel containing 17% acrylamide. Staining, destaining, and electroelution of the visualized fragments were done exactly as for the isolation of intact subunits.

Fig. III-5 shows the acid-cleaved fragments of the H subunit, after isolation by electrophoresis. No contamination is visible in either fragment. The molecular weights of the fragments were estimated by comparing their mobilities to the mobilities of water-soluble standards. This procedure gives estimated molecular weights of 17.5 kD and 11.8 kD. From the deduced amino acid sequence cleavage after Asp₁₄₈ should generate fragments of molecular weights 16.8 kD and 11.8 kD. For both fragments generated, the apparent molecular weights are very close to the expected values. It should be noted that the amino-terminal sequence of the 11.8 kD fragment must be determined before the assignment of the site of cleavage is definite. Nevertheless, the agreement between expected and observed molecular weights, and the fact that only one acid-labile site was observed after 24 h, provide an important verification of the deduced amino acid sequence.

c. M Subunit

Acid cleavage of the M subunit was harder to detect. This is

Fig. III-5. SDS-PAGE (17% acrylamide) of acid-generated fragments of the H subunit, isolated by preparative electrophoresis. Lane 1, molecular weight standards. Lane 2, chromatophore proteins (H and M are not resolved at this concentration of acrylamide). Lane 3, fragment with apparent molecular weight of 17.5 kD. Lane 4, fragment with apparent molecular weight of 11.8 kD.



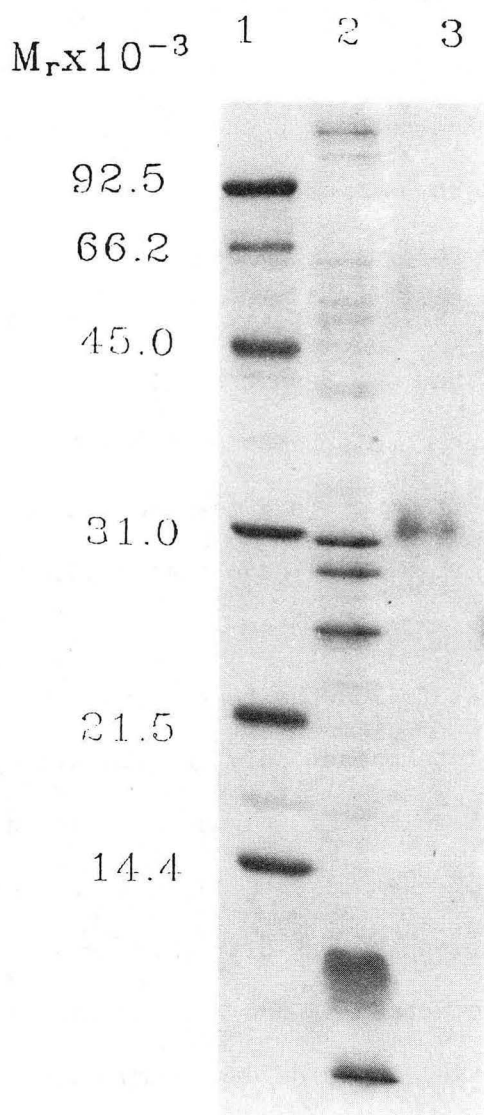
XBB 847-5637

Fig. III-5

primarily due to difficulties in staining the M subunit and its fragments. Although the stoichiometry of RC subunits is 1:1:1 (Okamura et al, 1974), the intensity of staining by Coomassie blue is always greater for the H subunit relative to that for M or L (see Figs. II-5 and II-10). L and M are also very difficult to detect by silver staining.

Another difficulty in studying the fragmentation of the M subunit is its "anomalous" mobility in SDS gels, as compared to water-soluble proteins. When chromatophores, native RC's, or the isolated subunit prior to ethanol precipitation are analyzed on SDS gels, the M subunit has an apparent molecular weight of 26-28 kD. (See Fig. III-1A, Fig. II-10, and Fig. III-1B, respectively, for examples of each type of sample.) The isolated M subunit after ethanol precipitation is shown in Fig. III-6. The apparent molecular weight after ethanol precipitation is 31.5 kD. The identity of the material shown in lane 3 of Fig. III-6 was confirmed by determining that the amino-terminal sequence was that of the M subunit (see Fig. III-2). The molecular weight deduced from the DNA sequence for the M subunit is 34.4 kD. The origin of the discrepancy between apparent molecular weight on SDS gels and deduced molecular weight is not known. In the case of RC subunits, the difference cannot be attributed to processing at the amino-terminus of the protein (see Fig. III-2). It is possible that a small portion of the L and/or M subunits are cleaved from the carboxy-terminus, leading to a smaller protein than predicted by the DNA sequence. This is not, however, the case for the M subunit from Rps. sphaeroides. For this subunit, the two carboxy-terminal amino acids were determined by digestion with carboxypeptidase A and

Fig. III-6. SDS-PAGE (15% acrylamide) of the M subunit after ethanol precipitation. Lane 1, molecular weight standards. Lane 2, chromatophore proteins. Lane 3, M subunit.



XBB 847-5640

Fig. III-6

shown to agree with the sequence deduced from the DNA (Williams et al, 1983).

The change in electrophoretic mobility upon ethanol precipitation has not been seen before to my knowledge. Ethanol precipitation may remove lipid which is still associated with the isolated subunit, or the ethanol may cause loss of some residual secondary structure still present in the SDS-denatured protein. Either of these processes might change the hydrodynamic properties of the SDS/protein particle migrating through the gel. The result of the differences between apparent molecular weights on gels and molecular weights deduced from DNA sequences is that one cannot be highly confident about an assignment of a fragment based on electrophoretic mobility alone. To date, no more definitive techniques (e.g measurement of amino acid compositions or amino-terminal sequences) have been applied to assign fragments generated from Rps. capsulata RC's.

Experiments to follow the time course of acid-cleavage of the M subunit could be interpreted only qualitatively. This was because detection of low levels of M fragments required extreme over-development of the silver-stained gel. Shortly after visualization, the entire gel blackened, rendering subsequent photographic reproduction of the gel impossible. An immediate photograph of the gel would have circumvented this problem.

After denaturation in 2% SDS at room temperature for 30 min formic acid was added to 90% final concentration. After 24 h at 35 C the sample was diluted 20 fold, lyophilized, redissolved and relyophilized, and analyzed electrophoretically in a gel containing 16% acrylamide. After silver-staining, two bands were visible with

apparent molecular weights of 32.0 kD and 24.0 kD. The deduced polypeptide sequence predicts that cleavage after Asp₈₀ would yield two fragments with molecular weights of 25.4 and 9.0 kD. The generation of a fragment with an apparent molecular weight of 24 kD (25.4 kD expected) suggests that cleavage did occur at Asp₈₀. No staining above background was visible in the region where a 9.0 kD fragment would be expected. After 24 hr in 90% formic acid the most prominent band to the eye was the 32 kD protein, which is probably the uncleaved M subunit.

The acid cleavage was repeated, using 80% formic acid for 40 h. Again, the electrophoretic analysis can be described only qualitatively. Proteins were seen with apparent molecular weights of 31.3 and 23.4 kD. After 40 h in 80% formic acid the smaller protein was stained more heavily than the larger one. As before, no staining was seen which would correspond to the amino-terminal fragment (9.0 kD) generated by cleavage at Asp₈₀.

Based on the suggestive results described above, preparative electrophoretic separation of acid-generated fragments from the M subunit was attempted. Incubation in 80% formic acid at 35 C was carried out for 48 h. After twice lyophilizing the sample, separation of the fragments was performed in a preparative SDS gel containing 16% acrylamide. The gel was stained for 20 min in 0.5% Coomassie blue and destained with continuous monitoring for the appearance of bands. After 30 min no staining pattern was visible. At this point, a test strip was cut from the gel and sectioned. The sections were analyzed electrophoretically. The regions of the preparative gel which were sectioned and analyzed corresponded to the

expected mobilities for polypeptides ranging from 40 kD to <5 kD. Fig.III-7 shows the silver-stained electrophoretogram obtained from the sections of the preparative gel. Only one band is visible above background, with an apparent molecular weight of 23.7 kD. (25.4 kD is expected from cleavage at Asp₈₀.) No bands are visible near 9 kD (expected amino-terminal fragment) or near 32 kD (unfragmented M subunit). The absence of staining at 32 kD suggests that the majority of the M subunit has been fragmented by treatment with 80% formic acid for 48 h. The absence of material at 9 kD is discussed below.

d. L Subunit

The deduced sequence for the L subunit contains no acid-labile sites, but contains three Glu's which should be cleavable by S. aureus V8 protease. Enzymatic digestion of the L subunit has been carried out, but not yet on a preparative scale. The analytical scale reactions suffer from the same drawback encountered with the M subunit. Detection of fragments by silver-staining usually required such over-development of the stain that the gel soon blackened. I will describe qualitatively the appearance of gels which were not photographed. As yet, no fragments have been isolated from the L subunit.

The L subunit, frozen in 0.1% SDS, 100 mM DTT, was warmed to room temperature. A solution of V8 protease (Miles Labs) dissolved in ammonium bicarbonate buffer, EDTA, pH 7.8 was added to give the following final concentrations; NH₄HCO₃, 75 mM; EDTA, 5 mM. Digestions were performed at 35 C, using protease to subunit ratios of approximately 1/5 and 1/10 (w/w). Aliquots were removed at 8, 20, and


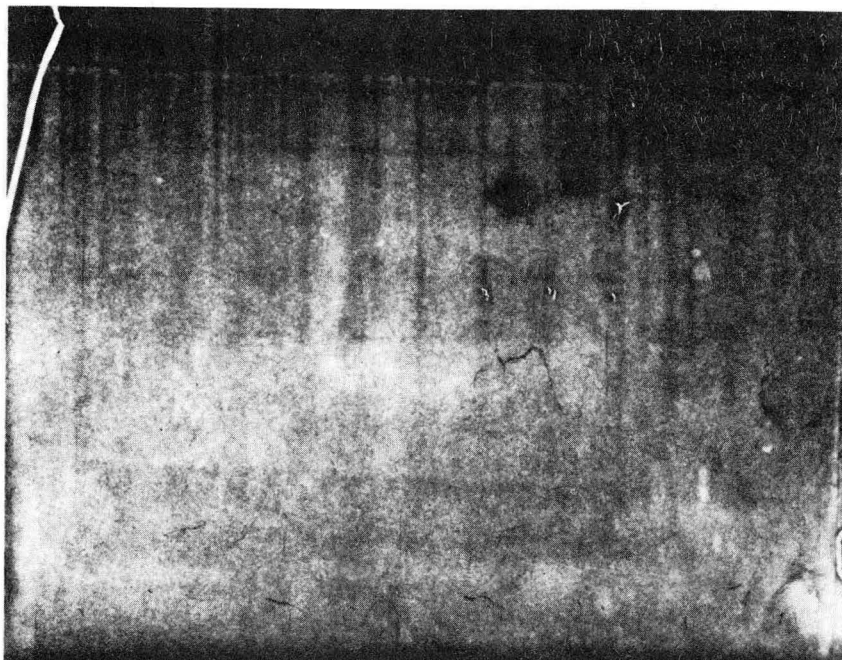


Fig. III-7. SDS-PAGE (17% acrylamide) of slices cut from a test strip of a preparative gel onto which was loaded the contents of an acid digestion of the M subunit (80% formic acid at 35 C for 48 h). The visible fragment has an apparent molecular weight of 23.7 kD.

apparent $M_r \times 10^{-3}$

23.7



XBB 847-5638

Fig. III-7

44 h and frozen until analyzed by SDS-PAGE.

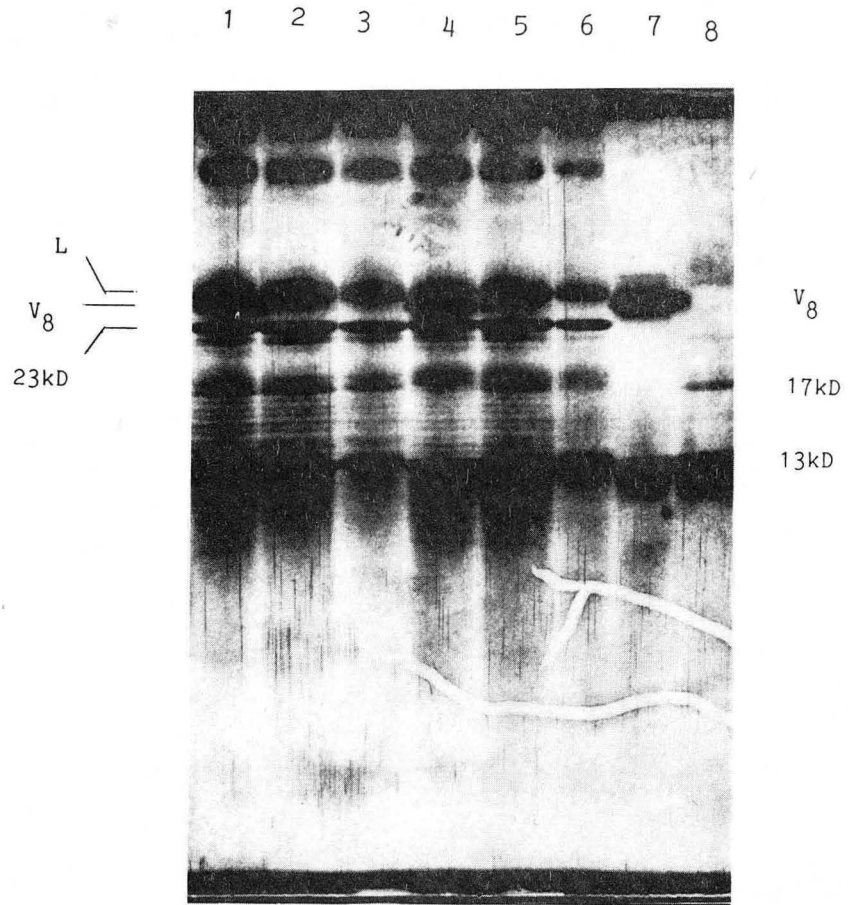
Fig. III-8 shows a silver-stained electrophoretogram of the digestion products obtained. Lanes 7 and 8 show the autodigestion products of the V8 protease, after 8 and 20 h. The undigested protein has an apparent molecular weight of 26.2 kD, very close to the value of 27 kD expected from the amino acid sequence (Drapeau, 1978). A prominent autodigestion product with an apparent molecular weight of 13 kD is also visible. A faint ladder pattern of bands (at least 7 bands) is visible on the original gel between 18 kD and 13 kD. The V8 protease contains 12 glutamic acid residues, so a large variety of autodigestion products is possible. After 20 h of autodigestion, essentially all of the V8 enzyme is degraded. In addition to the 13 kD fragment seen after 8 hr, another fragment with an apparent molecular weight of 17 kD is visible.

Lanes 1-3 of Fig. III-8 show the digestion products of the L subunit obtained after 8, 20, and 44 h using an enzyme to substrate ratio of 1/10. The bands with apparent molecular weights of 49 and 27.5 kD are assigned respectively as a dimer and monomer of the L subunit. Prior to EtOH precipitation, the L subunit has an apparent molecular weight of 25 kD (as in Fig. III-2). Thus ethanol precipitation alters the electrophoretic mobility of L as well as M. The DNA sequence for the L subunit predicts a molecular weight of 31.6 kD. Directly below the L subunit the undigested V8 enzyme is visible. A prominent fragment is visible whose apparent molecular weight is 23.2 kD. This fragment is completely absent in the autodigestion lanes, so it is assigned as a digestion product from the L subunit. Two bands are visible with apparent molecular weights between 21 and 23 kD. The autodigestion

experiment (20 hr, lane 8) shows a faint band which may correspond to one of these bands. A reliable assignment of the origin of these two bands (protease or L subunit) cannot be made at this time. The fragment with an apparent molecular weight of 17 kD, the fragments giving rise to the ladder pattern between 17 and 13 kD, and the fragment at 13 kD, are all assigned as autodigestion fragments of the V8 protease, because they are apparent in lanes 7 and 8. The three fragments migrating ahead of the 13 kD autodigestion fragment probably arise from autodigestion as well, since faint bands of similar electrophoretic mobility are seen in lanes 7 and 8. Longer digestion times (lanes 2 and 3) do not reveal more fragments of the L subunit, or even increased intensity in the 23 kD fragment. Staining intensity is lost in the bands assigned as L and a dimer of L, but it is not clear where the digested material is migrating. The loss of staining intensity is not due solely to heat-induced aggregation of the L subunit, which would lead to material which stays at the top of the gel. This can be seen by examining lanes 4, 5, and 6 of Fig. III-8, wherein the digestion was carried out using a protease/subunit ratio of 1/5. Especially, compare lane 6 to lane 3. The higher concentration of protease in lane 6 clearly results in a faster loss of staining intensity in the bands assigned to the L subunit, consistent with the proposal that the loss of staining intensity is due to digestion by the V8 enzyme. What remains unclear is what has happened to the liberated fragments. It may be that fragments smaller than 23 kD were generated but escaped detection.

At this point, only the fragment with an apparent molecular weight of 23 kD can be unequivocally assigned as arising from

Fig. III-8. SDS-PAGE (17% acrylamide) of digestion products from the L subunit, using S. aureus V8 protease. Lanes 1-3, protease/subunit = 1/10 (w/w). Lane 1, 8 h digestion. Lane 2, 20 h digestion. Lane 3, 44 h digestion. Lanes 4-6, protease/subunit = 1/5 (w/w). Lane 4, 8 h digestion. Lane 5, 20 h digestion. Lane 6, 44 h digestion. Lane 7, autodigestion products of the V8 enzyme, after 8 h. Lane 8, autodigestion products of the V8 enzyme, after 20 h. All digestions performed at 35 C.



XBB 849-6684

Fig. III-8

digestion of the L subunit. Examination of the deduced amino acid sequence for this subunit (Youvan et al, 1984b) reveals two potential cleavage sites which could give rise to the observed fragment. Cleavage after Glu₇₂ would generate two fragments with a molecular weights of 23.4 and 8.1 kD. Cleavage after Glu₂₁₂ would yield fragments of 23.6 and 7.9 kD. Clearly, gel electrophoresis cannot be used to distinguish which of these sites has been cleaved. Glu₇₂ is followed by an Asn residue. Glu₂₁₂ is followed by an Asp residue. Cleavage has been seen at Glu residues followed by both Asp and Asn (Houmard and Drapeau, 1972), so the specificity of the enzyme cannot be used to predict which site was cleaved. At this point it is not possible to state which site (Glu₇₂ or Glu₂₁₂) was cleaved. Amino acid composition or amino-terminal sequence data for the 23 kD fragment is needed before this assignment can be made.

A serious problem with interpreting the cleavage shown in Fig. III-8 is the presence of fragments derived from the V8 enzyme. Also, from lanes 2, 3, 5, 6, and 8 it is obvious that the large majority of the V8 enzyme is lost by 20 h, due to autodigestion. Both of these problems were addressed by using a total protease to substrate ratio of 1/30 (w/w), and adding the protease at 12 hr intervals. Each addition of protease corresponded to approximately 1% (w/w) of the weight of L subunit being digested. Additions were made at 0, 12, and 24 h. After 36 hr at 35 C the sample was analyzed by SDS-PAGE. Shortly after staining, bands were visible with apparent molecular weights of 51, 28.5, 23.0 and 15.5 kD. The most prominent band to the eye was the fragment at 23 kD, followed by the band at 28.5 kD. Both the fragment at 51 kD and at 15.5 kD were only slightly visible. The

band at 51 kD is probably a dimer of L, while the band at 28.5 kD is probably the uncut subunit. As discussed above, the fragment at 23 kD could arise from cleavage after Glu₇₂ or Glu₂₁₂. Cleavage at both of these sites would yield a fragment with a molecular weight of 15.5 kD. The fragment observed with an apparent molecular weight of 15.5 kD may be due to cleavage at both Glu₇₂ and Glu₂₁₂, but this is far from certain. An autodigestion product was seen with an apparent molecular weight of 17 kD (see lane 8, Fig. III-8). From gel to gel, enough variability in electrophoretic mobility is seen that the fragment with an apparent molecular weight of 15.5 kD may be the autodigestion product seen in lane 8, Fig. III-8. Based on mobilities alone, it is impossible to accurately assign the origin of the 15.5 kD fragment.

Sequential addition of the protease did increase the efficiency of cleavage. Without sequential addition of protease, 44 h at a protease/substrate ratio of 1/10 results in a majority of the subunit remaining uncut (see lane 3, Fig. III-7). With a protease/substrate ratio of 1/5, 44 h cleavage results in roughly half of the subunit appearing as a 23 kD fragment. With sequential addition of enzyme, 36 h cleavage at a protease/substrate ratio of 1/30 was sufficient to yield the 23 kD fragment as the major product visible on the gel.

Fragmentation of both M and L resulted in only one detectable fragment. In both cases, another fragment smaller than 10 kD was expected. The inability to detect these small fragments may be related to their size. The amount of stain bound in a given band is roughly proportional to the mass of protein present (discounting differences in amino-acid composition). Generation of equimolar amounts of two fragments would yield less mass of an 8-9 kD fragment,

as compared to a 23-25 kD fragment. Another difficulty in detecting fragments smaller than 10 kD may be related to the conditions of electrophoresis. Gels containing 16% acrylamide were used to try to minimize the diffusion of low molecular weight fragments during electrophoresis. A more effective technique would be the use of gels containing acrylamide concentration gradients. As proteins migrate through a gradient gel the mobility of the leading edge of the chromatographic zone continually decreases, due to the increasing acrylamide concentration. This results in a sharper zone which is more easily detected above the background staining.

The results presented for the M and L subunits are clearly preliminary. The data presented do demonstrate that M and L can be solubilized and denatured in SDS (+ DTT for L) as a first step in a fragmentation scheme. It remains to be seen whether this approach can be extended to yield smaller fragments of interest in studying the sites of cofactor binding.

4. CONCLUSIONS

The reaction center subunits H, M, and L were separated by preparative electrophoresis and electroelution. The amino-terminal sequences of each subunit were determined by automated Edman degradation. For all three subunits there is an exact correspondence between the sequence determined by Edman degradation and the sequence deduced from the DNA (Youvan et al, 1984a). Comparison of the sequences determined from the protein and from the DNA allows one to assess the extent of post-translational processing at the amino-terminus of each protein. The M and L subunits are processed

so as to remove the amino-terminal Met. No residues are removed from the amino-terminus of the H subunit. None of the subunits undergoes post-translational processing at the amino-terminus which is more extensive than removal of one amino acid.

The RC subunits were fragmented by adapting procedures developed for water-soluble proteins. The main modification was the use of SDS to both solubilize and denature the subunits. The H and M subunits were cleaved at Asp-Pro peptidyl bonds, using formic acid. Each subunit contains only one such bond. The L subunit was digested with the S. aureus V8 protease under conditions such that the protease cleaves after Glu residues.

Acid treatment of the H subunit results in two fragments. The two fragments were isolated by preparative electrophoresis. The apparent molecular weights of the fragments on SDS gels are 17.5 and 11.8 kD. The molecular weights expected from the DNA sequence for the H subunit are 16.8 and 11.8 kD. The number of fragments generated, and the molecular weights of the fragments, agree well with the results expected from the DNA sequence.

After acid treatment of the M subunit, only one fragment could be detected. The apparent molecular weight of this fragment on SDS gels was 23.7 kD. The DNA sequence for the M subunit predicts that acid treatment will generate two fragments with molecular weights of 25.4 and 9.0 kD. The apparent molecular weight of the one fragment detected is close to the expected molecular weight of the larger fragment, especially considering the difficulties of estimating molecular weights of hydrophobic proteins from electrophoretic mobilities. The inability to detect the

smaller fragment may be related to the conditions used to separate the products of acid treatment. Because only one of the expected fragments was detected, the site of cleavage by formic acid can only tentatively be assigned to the site predicted by the DNA sequence.

The L subunit was dissolved in 0.1% SDS, 100 mM DTT and digested with S. aureus V8 protease. One digestion fragment of 23.2 kD was detected. The DNA sequence of the L subunit predicts two detectable sites of cleavage by the V8 protease under the conditions used. Cleavage at one of these sites would generate a fragment of 23.4 kD. Cleavage at the other site would yield a fragment of 23.6 kD. It is not possible to determine which of these sites was cleaved to yield the fragment detected. No small fragment which would correspond to the complementary fragment to the 23.2 kD fragment was detected. As with the M subunit, the absence of the expected small fragment means that the protease digestion can be used only as a suggestive corroboration of the DNA sequence.

CHAPTER IV. INVESTIGATIONS OF QUINONES IN REACTION CENTERS
AND PHOTOSYSTEM II

1. INTRODUCTION

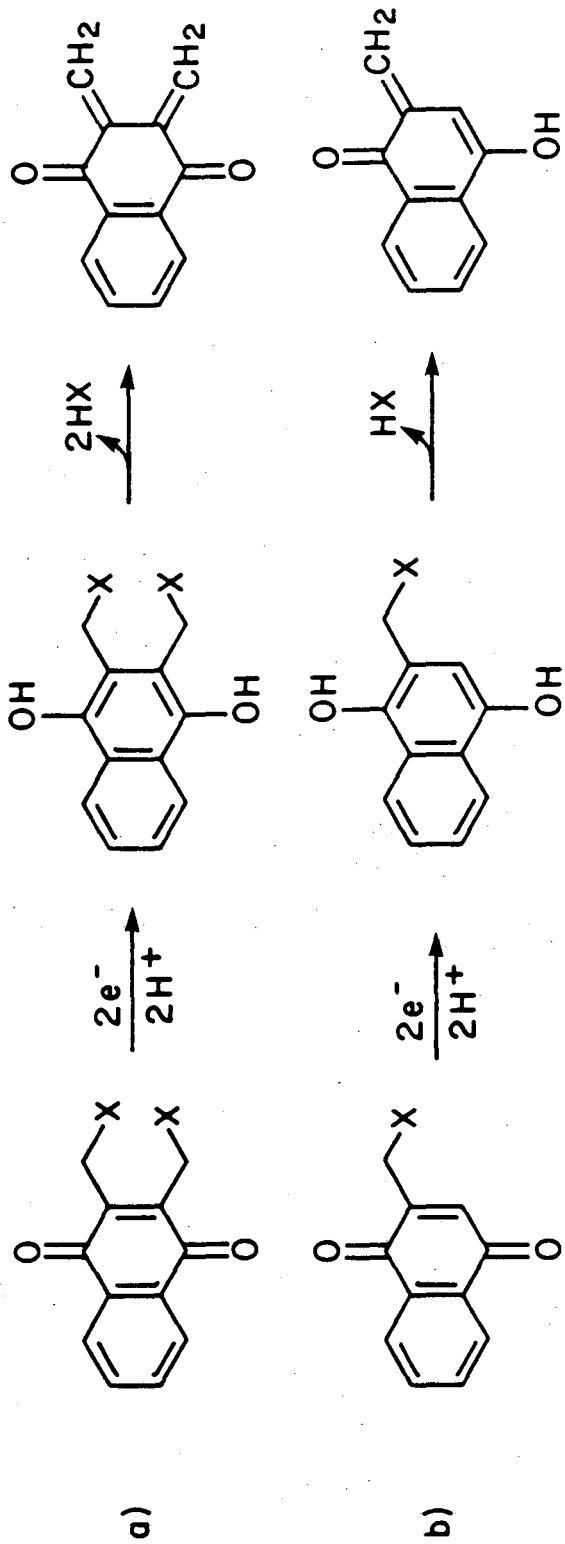
Substituted benzoquinones serve as electron acceptors in bacterial reaction centers (RC's) and photosystem two (PSII) of higher plants. RC's from Rps. sphaeroides and Rps. capsulata contain ubiquinone-10 (UQ-10). The quinone in PSII from spinach is plastoquinone-9 (PQ-9). The two-electron gate model (see Chap. I, part 2 and Wraight, 1982) has been used to describe electron transfer reactions at the acceptor side of RC's and PSII. One of the most striking features of these electron transfer reactions is the stabilization of the second electron acceptor, Q_b , in the semiquinone anion form. This stabilization is probably due to interactions between the semiquinone and the protein to which it is bound. Sequence homologies have been found between the Q_b apoprotein of PSII and the L and M subunits of RC's (Williams et al, 1983 and Youvan et al, 1984b). The homologous sequences may be involved in binding quinones and stabilizing Q_b in the semiquinone form (Hearst and Sauer, 1984).

One approach towards studying the interactions between quinones and RC's is to extract the quinones from RC's and then reconstitute the quinone-binding sites with quinones, either physiological or non-physiological. Extraction and reconstitution studies using RC's from Rps. sphaeroides, strain R-26, have established that RC's bind two molecules of UQ-10 (Okamura et al, 1975). Only one molecule of UQ-10 need be bound to stabilize charge separation. These same studies investigated the reconstitution of

RC's with several quinones other than UQ-10. Of the non-ubiquinone acceptors tested, the best analogue to UQ-10 (in terms of affinity of binding and preservation of electron transfer kinetics) was menadione (2-methyl-1,4-naphthoquinone). Extraction of all UQ-10 from the RC permitted reconstitution with azido-anthraquinone, which was shown to restore photochemistry (Marinetti et al, 1979). After UV irradiation of RC's reconstituted with azido-anthraquinone, a covalent bond was formed between the RC M subunit and the anthraquinone. From this observation the authors concluded that the primary quinone binding site was on or very close to the M subunit.

"Bioreducible quinones" is the name given to a class of quinone derivatives which have been investigated as anti-tumor agents (reviewed in Lin et al, 1976). The common structural feature of bioreducible quinones is the attachment of one or more potential leaving groups adjacent to the aromatic ring. Moieties which have been used as leaving groups include halides, methyl sulfonates, and acetate groups. Bioreducible quinones with one or two leaving groups have been investigated. Sartorelli and co-workers have proposed the reaction scheme shown in Fig. IV-1 as the mechanism of action of bioreducible quinones. Upon reduction and subsequent departure of the leaving group(s) a quinone methide is formed. This structure is a good alkylating agent and is believed to be the reactive intermediate involved in anti-tumor activity. Several lines of evidence support this idea. The anti-tumor activity of mitomycin C (a prototype bioreducible quinone) is dependent on reduction of the benzoquinone ring (Schwartz et al, 1963). Bioreducible quinones were reduced in vitro with NaBH_4 and the

Fig. IV-1. Proposed mechanism for the reductive activation of bioreducible quinones. (From Sartorelli et al, 1984).



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Fig. IV-1

reaction mixture treated with aniline and morpholine. The products isolated were those expected if reduction of the quinone produced the reactive quinone methide (Lin and Sartorelli, 1973).

Bioreducible quinones have been shown to inhibit NADH and succinate dehydrogenases (both quinone reductases), but it was not demonstrated that the inhibition was due to alkylation of the enzyme (Lin et al, 1973).

I find the term "bioreducible quinone" somewhat ambiguous, since there are many quinones which are reducible by RC's but which never act as alkylating agents. Consequently, I will refer to quinones which possess active leaving groups, and thus may serve as alkylating agents, as potential suicide quinones. A suicide inhibitor is a substrate or co-factor analogue which is capable of entering into the catalytic cycle of an enzyme (as opposed to an inhibitor which occupies a binding site but never reacts). The catalytic action of the enzyme is responsible for activating a suicide inhibitor, which subsequently irreversibly inhibits the enzyme. By catalyzing a reaction with this type of inhibitor an enzyme "kills itself", hence the name suicide inhibitor.

In this chapter I will describe procedures for the extraction of UQ-10 from RC's isolated from Rps. capsulata, strain KZR8A1. The procedures were adapted from the methods used to extract UQ-10 from RC's isolated from Rps. sphaeroides, strain R-26 (Okamura et al, 1975). Methods will be described for extraction of only Q_b or of both Q_a and Q_b . Reconstitution of the quinone binding sites has been carried out with UQ-10 and 2,3-bis(acetoxymethyl)-1,4-naphthoquinone (compound III, see Fig. IV-10), a potential suicide

quinone. The effects of these reconstitutions were assayed by bleaching of the primary donor, P_{850} . Several potential suicide quinones were tested for their ability to inhibit oxygen evolution in active PSII particles. PSII particles were used as a model system to test suicide inhibition because of the ease of measuring rates of O_2 evolution before and after inhibition. The goal of this comparative study was to identify potentially useful suicide quinones to be used in identifying quinone binding regions of quinone reductases (e.g. RC's and PSII).

2. EXTRACTION OF QUINONES FROM BACTERIAL REACTION CENTERS

a. Methods

To extract Q_b , RC's were diluted with 10 mM Tris-HCl, 0.05% LDAO, pH 8.2 (buffer) to give an absorbance at 800 nm of 0.6 cm^{-1} . LDAO was added to a final concentration of 0.6% and the RC's were incubated at room temperature for 4 h in the dark. The sample was cooled to 4 C and applied to a cyt c column pre-equilibrated with buffer. (See Chap. II for general procedures concerning isolation of RC's on the affinity column). The column was washed with sufficient buffer to reduce the absorbance of the eluent at 760 nm (BChl extracted from the RC's by detergent) to $<0.02 \text{ cm}^{-1}$. The quinone-depleted RC's were eluted from the cyt c column in buffer containing 300 mM NaCl and dialyzed to remove the NaCl.

To extract both quinones, RC's were diluted as above and incubated in 1.0% LDAO, 5 mM o-phenanthroline at room temperature for 4 h in the dark. After cooling, the sample was applied to the affinity column. The column was washed as above, and RC's eluted

with 300 mM NaCl.

To assay the results of a particular extraction, it is necessary to measure the occupancy at both the Q_a and Q_b sites. A quinone must be bound at the Q_a site to stabilize charge separation (Okamura et al, 1975), so the ability to photobleach P_{850} was used as an assay of occupancy at the Q_a site. In RC's with a full complement of Q_a , approximately 85% of the 850 nm band is bleachable (see Fig. II-13, and Feher, 1971). Thus the occupancy at the Q_a site was estimated by equation 1:

$$\text{occupancy} = (\text{bleaching at 850 nm/absorbance at 850 nm})/0.85. \quad (1)$$

As mentioned in Chap. II, it is very difficult to get a saturated, yet fully reversible, bleaching signal from RC's purified from strain KZR8A1. Therefore, full bleaching was estimated by double reciprocal plots as shown in Fig. II-13.

The bleaching of RC's is accomplished by means of actinic illumination at 595 nm (see Chap. II). When this actinic illumination is turned off, the absorbance at 850 nm is recovered. The kinetics of recovery depend on the position of the electron during illumination. If only Q_a is present, the relevant reaction is $P^+Q_a^- \rightarrow PQ_a$. The rate constant for this first order back reaction is 10 s^{-1} . When Q_b is present, it can accept electrons from Q_a^- . In this case, when the light is turned off the relevant back reaction is $P^+Q_aQ_b^- \rightarrow PQ_aQ_b$. The first order rate constant for this reaction is 1 s^{-1} . Thus the kinetics of the P_{850} recovery can be used to estimate the occupancy at the Q_b binding site. If

the Q_b site is fully occupied, one expects monophasic recovery kinetics. From the rate constant (1 s^{-1}), the recovery after one second should be 63%, while the recovery after two seconds should be 86%. If the Q_b site is entirely empty, one again expects monophasic recovery kinetics. In this case the recovery should be 100% in one second. The measurements of recovery kinetics were made in a Cary 14 spectrophotometer, so it was not possible to reliably measure recoveries at times less than one second. 100% recovery in one second was taken to indicate no binding at the Q_b site.

A more general case is a heterogeneous RC sample in which the Q_b occupancy is between 0 and 100%. In this case one expects biphasic recovery kinetics. RC's which contain a bound Q_b should recover at a rate of 1 s^{-1} , while RC's which do not contain a bound Q_b should recover at a rate of 10 s^{-1} . By estimating the amplitudes of the two kinetic components, one can assay the occupancy at the Q_b site. An equivalent method is to measure the extent of recovery at several times, and compare these numbers to values expected for different occupancies at the Q_b site. Table IV-1 shows the expected recoveries for different occupancies at Q_b . This table was constructed by assuming that all the RC's in a sample act independently of each other, and that the bleaching of both classes of RC's (those with or without Q_b) is saturated. If the last assumption is not met, then the occupancy at the Q_b site will be overestimated, because it will be easier to bleach RC's which contain Q_b bound.

From the table, it can be seen that to reliably assay the Q_b occupancy of an RC sample which contains less than two bound

Table IV-1. Expected kinetics for the recovery of the bleaching at 850 nm, as a function of the Q_b occupancy. See text for assumptions required to construct this table.

Recoveries Expected at Various Times for
 Different Q_b Occupancies (expressed as
 percentage of complete recovery)

Q_b occupancy	time						
	100 ms	250 ms	500 ms	750 ms	1 s	2 s	3 s
	0%	63	92	99	100	100	100
10%	58	85	93	95	96	99	100
20%	53	80	87	91	93	97	98
40%	42	64	75	81	85	95	98
60%	31	50	63	72	80	92	97
80%	20	36	51	62	71	89	96
90%	15	29	45	58	67	88	96
100%	10	22	39	53	63	87	95

Table IV-1

quinones/RC, it is necessary to have kinetic data for times shorter than one second. This is not possible in the Cary 14, but recent results using an Aminco spectrophotometer indicate that this type of kinetic analysis can be used to assay Q_b occupancy in samples containing considerably less than two quinones bound per RC (Maxson, unpublished observations). Using various UQ-10/RC ratios, the Q_b occupancies calculated by the kinetic method are consistent with one binding constant for the Q_b site, and the binding constant calculated is very similar to the constant obtained with RC's from Rps. sphaeroides, strain R-26 (Okamura et al, 1982b and Wraight, 1977). These observations demonstrate the validity of using a kinetic analysis to assay occupancy of the quinone binding sites.

In this work, RC's containing UQ-10 were prepared such that the Q_b site was fully occupied or nearly empty. These two cases are readily distinguishable because the recovery kinetics differ by an order of magnitude. Also, the samples are homogeneous with respect to Q_b occupancy. This means that it is not necessary to consider the saturation behavior of two classes of RC's.

b. Results and Discussion

Fig. IV-2 shows the near-IR absorption spectrum of RC's which were extracted with 0.6% LDAO. This spectrum is the same as seen for RC's not treated so as to remove Q_b (see Fig. II-11), indicating that the pigment-protein and pigment-pigment interactions responsible for the IR spectrum have not been altered by the extraction procedure. The recovery of RC's from this procedure is approximately 85%. The fraction of the RC's not recovered is

Fig. IV-2. Near-IR spectrum of reaction centers from Rps.
capsulata, strain KZR8A1. Reaction centers were extracted with
0.6% LDAO to remove Q_b .

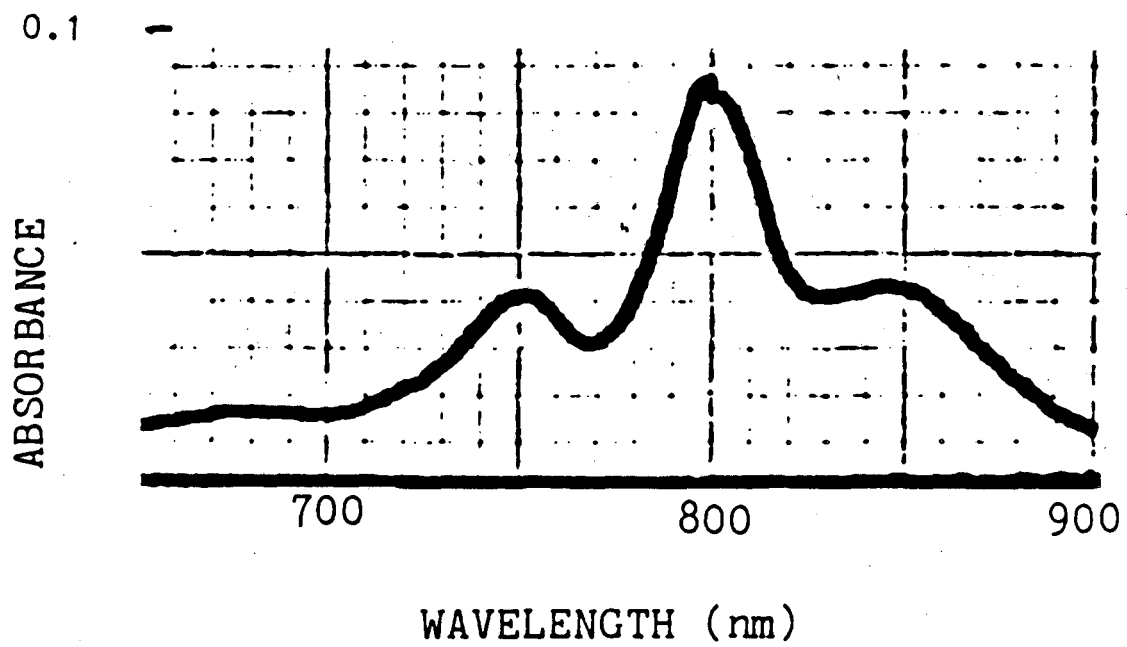


Fig. IV-2

probably denatured by the detergent. During the loading of RC's on the cyt c column, material with an absorbance maximum at 760 nm eluted. This material is probably free BChl extracted from the RC's by the LDAO.

For RC's extracted with 0.6% LDAO, extrapolation of the bleaching signal to infinite light intensity gives a bleaching at 850 nm equal to 92% of the total absorbance at 850 nm. By equation 1, this is equivalent to a 108% occupancy of the Q_a binding site. An occupancy of greater than 100% is not physically meaningful. I interpret this data to indicate that the Q_a site is fully occupied. I attribute the value greater than 100% to error in extrapolating the bleaching to infinite light intensity. Typically, the double reciprocal plots were constructed from bleaching assays which were 30-40% saturated at full light intensity (see Fig. II-13).

The occupancy of the Q_b site was assayed by recording the kinetics of recovery of absorbance at 850 nm. Fig. IV-3 shows such a kinetic trace. Within one second of turning off the light the absorbance is fully recovered. (It is not possible to get reliable recoveries at less than one second with the Cary 14.) From Table I, a recovery of 100% within one second is indicative of a sample containing no Q_b . From the size of the bleaching signal in Fig. IV-3 (5×10^{-3} absorbance units) and the size of the noise on the Cary 14 (approximately 3×10^{-4} absorbance units), I estimate that I could not distinguish 100% recovery within one second from 95% recovery. A 95% recovery in one second corresponds to a Q_b occupancy of 15%. I conclude that the RC's extracted with 0.6% LDAO contain 0-15% Q_b .

Fig. IV-4 shows the near-IR spectrum of RC's extracted with 1.0%

Fig. IV-3. Bleaching and recovery kinetics of reaction centers from KZR8A1 after extraction with 0.6% LDAO. Bleaching recorded at 850 nm. Arrows mark points at which actinic light was turned on and off.

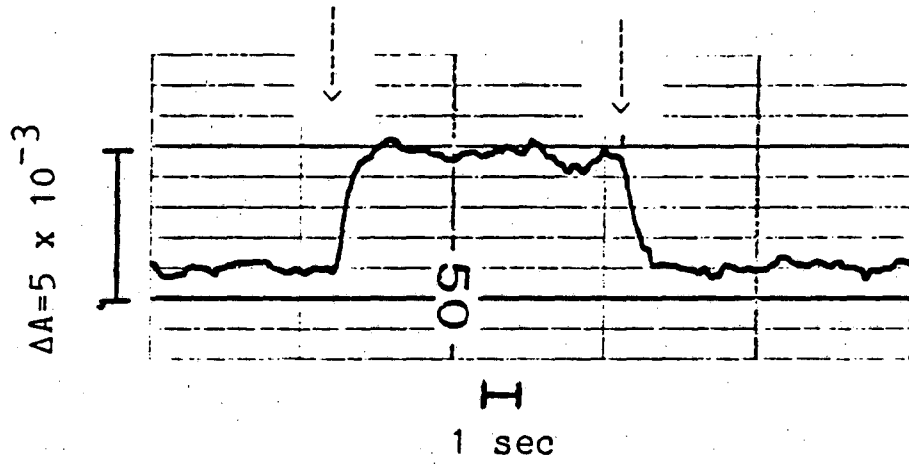


Fig. IV-3

LDAO and 5 mM o-phenanthroline. The spectrum is essentially the same as that of unextracted RC's. Again, no alteration of the pigment environment during the extraction procedure is indicated. The yield of RC's for this extraction procedure was approximately 70%. Free BChl was observed eluting from the cyt c column during loading. The lower yield of RC's, as compared to the procedure to remove Q_b , is probably due to the higher concentration of LDAO used to extract both quinones.

For RC's extracted with 1.0% LDAO and 5 mM o-phenanthroline, extrapolation of the bleaching signal to infinite light intensity gives a $\Delta A_{850}/A_{850}$ of 5%, indicating an occupancy at the Q_a site of 6%. The small bleaching signals seen recovered completely within one second (Fig. IV-5), suggesting that no quinones are bound at the Q_b site. In RC's from Rps. sphaeroides, strain R-26, the affinity of the Q_a site for UQ-10 is at least five fold greater than the affinity of the Q_b site (Okamura et al, 1982b). Therefore, after extraction of 94% of the UQ-10 from the Q_a site, one would not expect any significant occupancy of the Q_b site, consistent with the fast recovery kinetics seen.

The results presented here for extraction of UQ-10 from RC's from Rps. capsulata, strain KZR8A1, can be compared to the results of Okamura and co-workers using RC's from Rps. sphaeroides, strain R-26 (Okamura et al, 1975). These workers adjusted the concentration of RC's to give an A_{800} of 2.0 prior to extraction. At this concentration, extraction with 2% LDAO was required to yield RC's containing 15% Q_b occupancy. (In my work extraction with 0.6% LDAO, at an $A_{800}=0.6$, gave 0-15% Q_b occupied.) The fact that a

Fig. IV-4. Near-IR spectrum of reaction centers from KZR8A1.
Reaction centers were extracted with 1.0% LDAO, 5 mM o-phenanthroline
to remove both quinones.

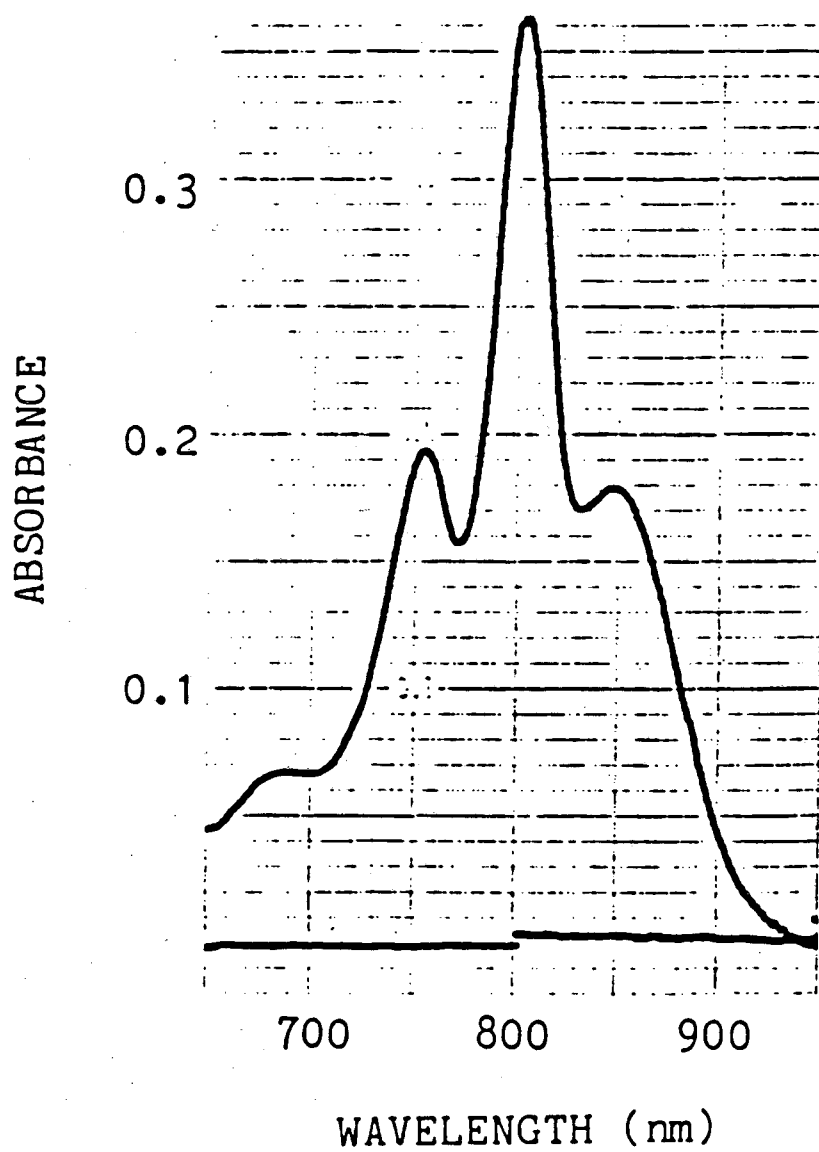


Fig. IV-4

Fig. IV-5. Bleaching and recovery kinetics of reaction centers from KZR8A1 after extraction with 1.0% LDAO, 5 mM O-phenanthroline. Bleaching recorded at 850 nm. Arrows mark positions at which actinic light was turned on and off.

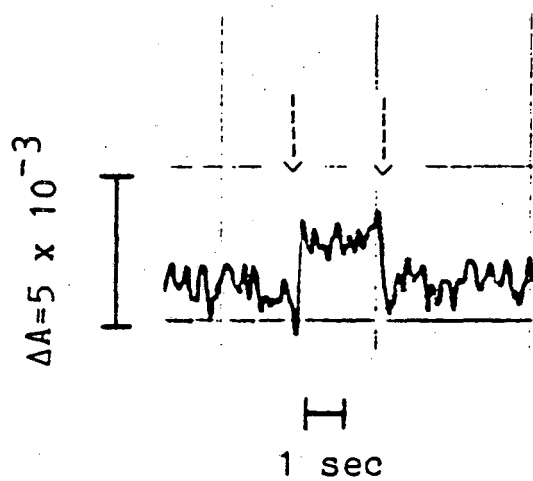


Fig. IV-5

lower LDAO concentration was as effective in extracting Q_b in my studies is probably due to the lower RC concentration used for the extraction. It is interesting to note that the RC/LDAO ratio is the same in both studies, and approximately the same extent of extraction of Q_b was obtained. To extract Q_a , Okamura and co-workers suspended RC's to an A_{800} of 0.2. At this RC concentration, 1.0% LDAO and 10 mM o-phenanthroline were used to extract greater than 90% of Q_a . In my work, RC's at an $A_{800}=0.6$ were extracted with 1.0% LDAO and 5 mM o-phenanthroline. From a double-reciprocal plot, I estimate that I extracted approximately 95% of Q_a . It should be noted that Okamura estimated the amount of Q_a bound by using radioactively labelled UQ-10. This procedure is probably more accurate than my procedure of extrapolating bleaching to infinite light intensity.

3. Reconstitution of Quinone Binding Sites

a. Methods

Quinones which were to be added back to RC's were dissolved in acetone. Acetone was chosen because of the very high solubility (>10 mM) of quinones in acetone. This meant that only a small volume of solvent needed to be added back to achieve high quinone/RC ratios. In all cases, the final concentration of acetone in the reconstituted RC samples was less than 0.2% (v/v). After addition of the quinones in acetone, the samples were maintained at room temperature for 3 h in the dark. At this point RC's were assayed for quinone binding or stored at 4 C. RC's in the presence of acetone and excess quinone were stored at 4 C for several days with

no visible degradation of the absorption spectrum or alteration in the bleaching behavior.

b. Results

RC's which were essentially devoid of Q_a and Q_b (Q_a occupancy $\leq 10\%$) were reconstituted with a ten-fold excess of UQ-10. The bleaching signal extrapolated to infinite light intensity was 95% of the absorbance at 850 nm. According to equation 1, this corresponds to an occupancy at the Q_a site of 112%. As before, I attribute the excess over 100% occupancy to error in extrapolating the bleaching to infinite light intensity.

Fig. IV-6 shows the kinetics of recovery of absorbance at 850 nm.

At one second, the recovery is 67%, indicative of an occupancy of 90% at the Q_b site (see table I). At two seconds, the recovery is 85%, indicative of a 100% occupancy. At three seconds, the recovery is 87%. This recovery is less than expected after three seconds, even for RC's containing 100% Q_b bound. The inconsistencies in the Q_b occupancy calculated from different time points indicates that the recovery is not truly monophasic.

Superimposed on the one second recovery component is a much slower component. This component may represent recovery in RC's in which an electron has been transferred to the detergent, lauryldimethylamine-N-oxide (LDAO). The amine oxide is capable of accepting electrons. The presence of the slow kinetic component makes it more difficult to analyze the recovery kinetics in terms of occupancy at the Q_b site. The amplitude and kinetic constant of the slow phase were analyzed by looking at times between 12 and 30 s.

Fig. IV-6. Bleaching and recovery kinetics after quinone-depleted reaction centers were reconstituted with a 10/1 molar excess of ubiquinone-10. Bleaching recorded at 850 nm. Arrows mark positions at which actinic light was turned on and off.

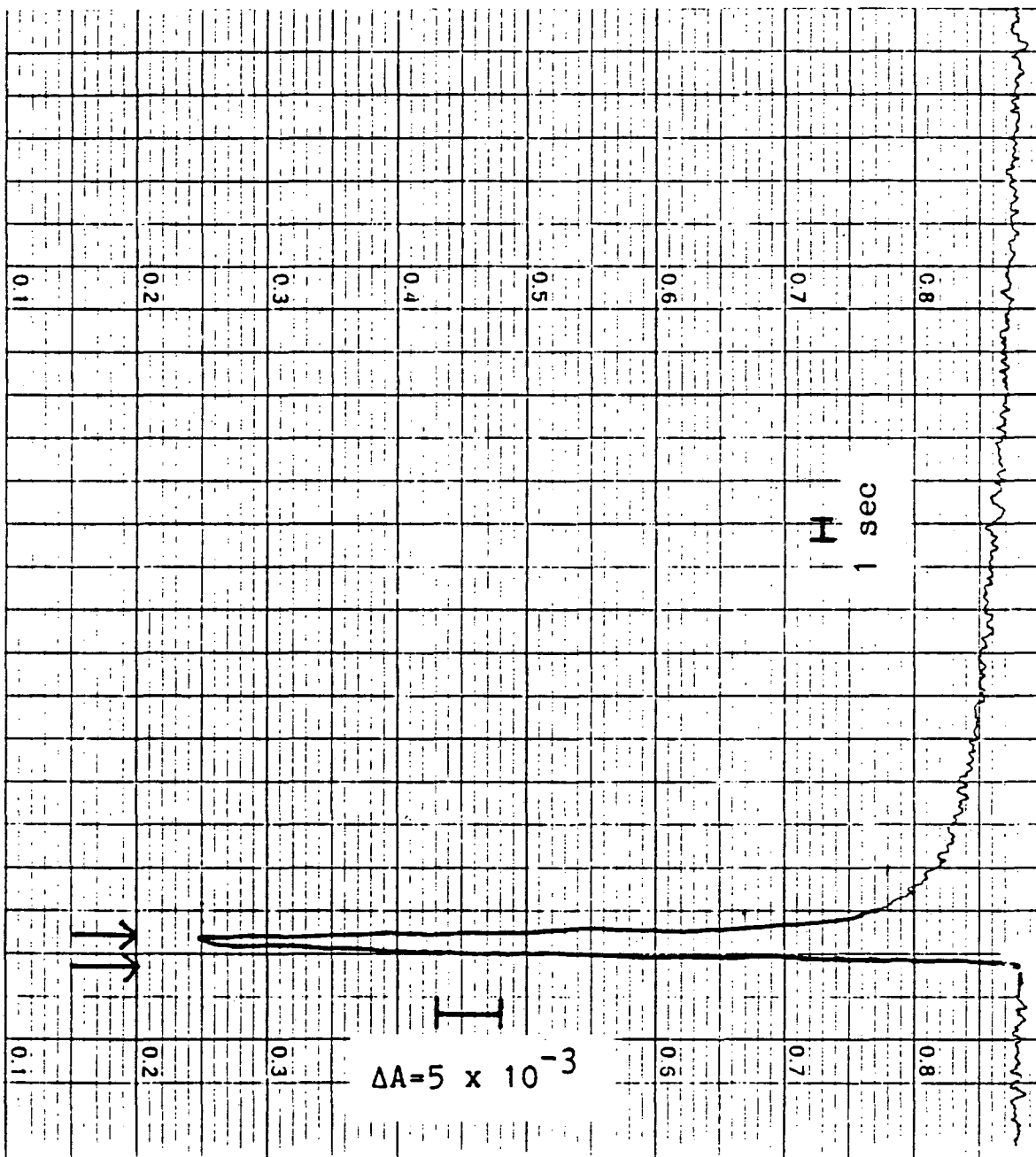


Fig. IV-6

The rationale for this choice of times is that after 12 s, any component with a recovery constant of 1 s^{-1} (back transfer from Q_b) should be negligible. Fig. IV-7 shows a plot of $\ln \Delta A / \Delta A_0$ vs. time for the period 12-30 s. The linear relation between $\ln \Delta A / \Delta A_0$ and time indicates that there is only one significant kinetic component in this time interval. The rate constant calculated from this plot is 0.055 s^{-1} . The amplitude of this component was estimated by extrapolating the signal at 12 s back to time zero, using the rate constant of 0.055 s^{-1} . The relative amplitude of this component is 10% of the total bleaching signal seen. When Q_b was absent, there was little or none of the very slow kinetic phase in the recovery kinetics (see Fig. IV-3). I suggested above that the very slow component may represent electron transfer from reduced quinone to LDAO. If this is the case, the absence of the very slow phase in RC's which contain only Q_a (see Fig. IV-3) suggests that the transfer to LDAO from Q_b^- is much more facile than transfer from Q_a^- . Therefore, the very slow component may represent RC's which contain the Q_b site occupied. From the recovery at one s (67%), I place a lower limit on the occupancy at the Q_b site at 90%. By assuming that the very slow component does represent RC's with a bound Q_b , I place an upper limit on the occupancy of the Q_b site of 100%. Thus reconstitution of RC's with a ten-fold molar excess of UQ-10 over RC's resulted in full occupancy at the Q_a site, and 90-100% occupancy at the Q_b site. These results are very similar to those of Okamura (Okamura et al, 1975). These workers found that reconstitution of quinone-depleted RC's with a tenfold excess of UQ-10 resulted in full occupancy at the Q_a site, and 95% occupancy at the Q_b site.

Fig. IV-7. Analysis of very slow kinetics of the recovery shown in Fig. IV-6. ΔA_0 is bleaching 12 sec after actinic illumination was turned off.

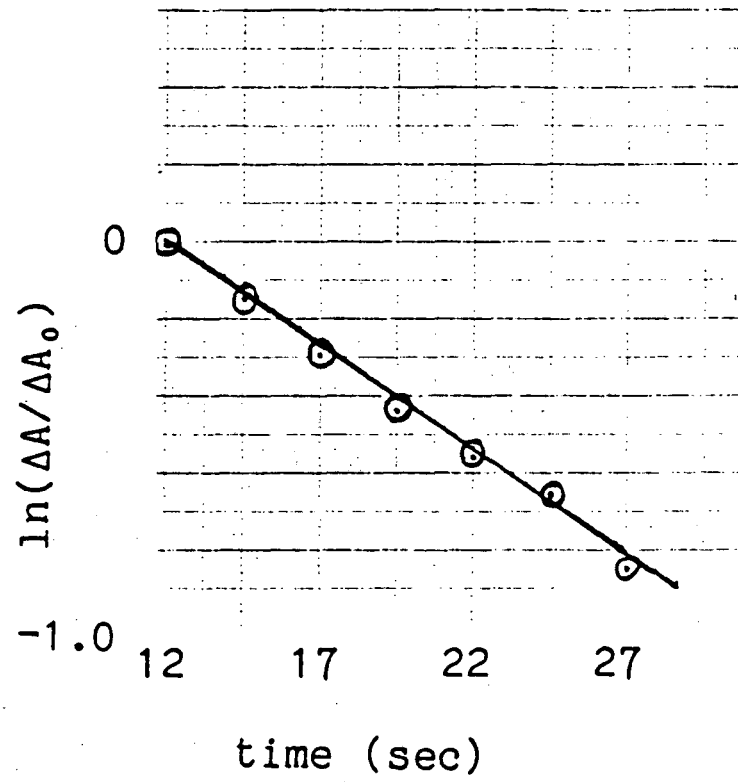


Fig. IV-7

RC's which were devoid of quinones were also reconstituted with a potential suicide quinone. The quinone used was 2,3-bis(acetoxymethyl)-1,4-naphthoquinone (compound III), kindly provided by Dr. A. Sartorelli, Yale Dept. of Pharmacology. I have no information about the binding constant of this compound to the quinone binding sites of RC's. The binding constants of menadione and phyloquinone (both 1,4-naphthoquinone derivatives) have been estimated from reconstitution experiments (Okamura et al, 1982b). Both compounds have affinities for the Q_a site which are experimentally indistinguishable from the affinity of UQ-10 for this site. The affinity of the naphthoquinone derivatives for the Q_b site is at least 10-fold less than the affinity of UQ-10 for this site. Based on these results, RC's were reconstituted with a 100-fold molar excess of compound III (ten times the molar ratio needed to occupy both sites with UQ-10).

The RC's reconstituted with the naphthoquinone derivative are bleachable, indicating that this quinone can occupy the Q_a site of RC's. Extrapolation of bleaching to infinite light intensities gave a saturated bleaching of 80% of the total absorbance at 850 nm. According to eqn. 1 this corresponds to an occupancy at the Q_a site of 94%. In light of the fact that I have estimated occupancies as high as 112% (a physically unreasonable result), an occupancy of 94% is indistinguishable by my techniques from an occupancy of 100%. The kinetics of recovery of absorbance at 850 nm are shown in Fig. IV-8. (The time scale is considerably longer in this trace than in Fig. IV-6). The kinetics of recovery shown in Fig. IV-8 were analyzed by plotting $\ln(\Delta A/\Delta A_0)$ vs. time (see Fig. IV-9). From

Fig. IV-8. Bleaching and recovery kinetics after quinone-depleted reaction centers were reconstituted with a 100/1 molar excess of 2,3-bis(acetoxymethyl)-1,4-naphthoquinone. Bleaching recorded at 850 nm. Arrows mark positions at which actinic illumination was turned on and off.

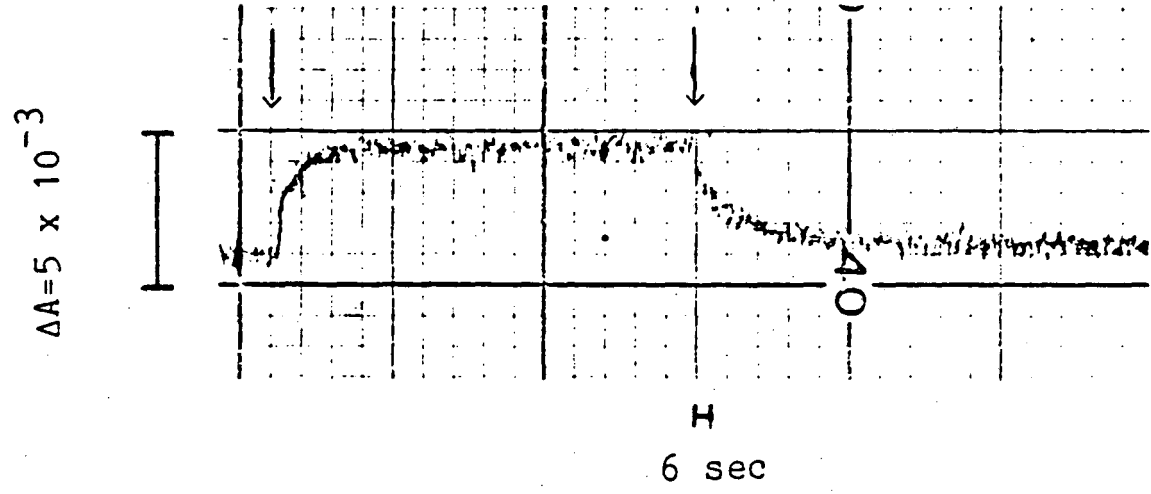


Fig. IV-8

Fig. IV-9. Analysis of recovery kinetics of bleaching signal shown in Fig. IV-8. ΔA_0 is bleaching signal when illumination was turned off. Linear regression analysis performed after excluding $t=0$ point. (Between 0 and 12 sec, at least one fast kinetic component in addition to the very slow component is present.)

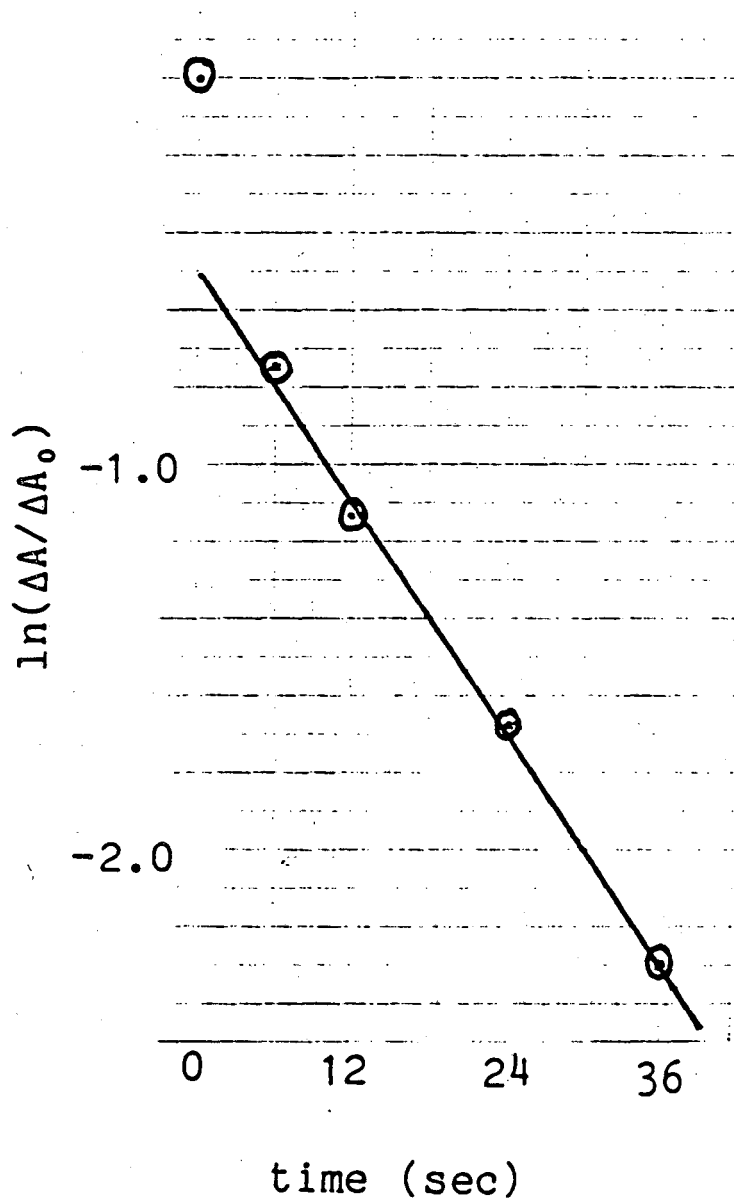


Fig. IV-9

Fig. IV-9 it is clear that the recovery is not monophasic. From the data available, it is not possible to extract a rate constant for the fast phase(s) of the recovery in Fig. IV-8. The rate constant for the very slow phase (times greater than five seconds) is obtainable from Fig. IV-9 by excluding the $t=0$ point from the linear regression analysis. The rate constant calculated for the time interval 6-24 sec is 0.051 s^{-1} . The relative amplitude of the very slow phase is approximately 60% of the total bleaching seen.

The origin of the heterogeneous recovery kinetics shown in Figs. IV-8 and IV-9 is not known. The fast and slow kinetic components may reflect electron transfer from naphthoquinone bound at the Q_a and Q_b sites, respectively, but if this is the case transfer from Q_b site is 20x slower than when this site is occupied by UQ-10. The rate of the very slow phase (0.051 s^{-1}) is almost identical to the very slow rate (0.055 s^{-1}) seen in RC's reconstituted with UQ-10. This suggests that the same process (e.g. back reaction from reduced LDAO) gives rise to both very slow phases. It should be noted that the time of illumination was quite different in the experiment with compound III, as compared to the experiment with UQ-10. With compound III, the illumination was continued for 2.4 min, and a relative amplitude of 60% was obtained for the very slow component. In the experiment with UQ-10, the illumination was only continued for 2 s, and the relative amplitude of the very slow component was 10%. If the very slow component arises in RC's in which an electron has been transferred to LDAO, it is reasonable that the amplitude of the slow component increases with time of illumination. Presumably an electron is transferred to

LDAO from Q_b^- (the slow phase was not seen in RC's containing only Q_a). The longer the RC is maintained in the state $P+Q_b^-$ (i.e. the longer the time of illumination) the more likely it is that an electron is transferred to LDAO. If the slow kinetic component arises only in RC's containing a quinone bound at the Q_b site, then at least 60% of the RC's reconstituted with a 100 fold excess of compound III contain an occupied Q_b site. It is definitive that reconstitution of RC's with a 100 fold excess of compound III results in an occupied Q_a site (94-100% occupancy). The results suggest, but do not prove, that this ratio of quinone/RC also leads to binding at the Q_b site.

4. Inhibition of PSII by Potential Suicide Quinones

a. Methods

O_2 -evolving PSII particles (kindly donated by Dr. N. Blough, U. C. Berkeley) were prepared as described previously (Blough and Sauer, 1984). The preparation method is essentially a hybrid of two published procedures (Kuwabara and Murata, 1982 and Berthold et al, 1981). The conditions of detergent extraction are as described by Kuwabara and Murata, while the buffers and pH used were those of Berthold et al. Inhibition studies with potential suicide quinones were carried out with PSII particles which had uninhibited activities of approximately $400 \mu\text{moles } O_2 \text{ evolved mg Chl}^{-1} \text{ h}^{-1}$. O_2 evolution measurements were made at Chl concentrations of 5-10 $\mu\text{g Chl/ml}$. Dichlorobenzoquinone (DCBQ) was used as the electron acceptor. Doubling the concentration of DCBQ had no effect on the O_2 evolution rates, indicating the assay was saturated with respect to

electron acceptor. All assays were performed with such a concentration of PSII particles that O_2 evolution rates increased linearly with amount of PSII particles added.

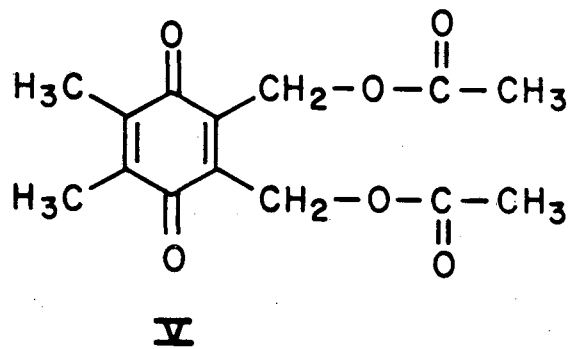
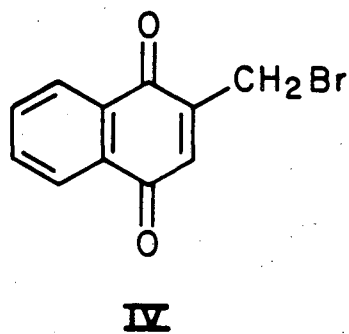
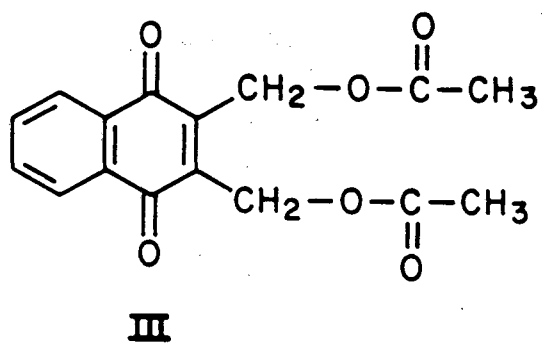
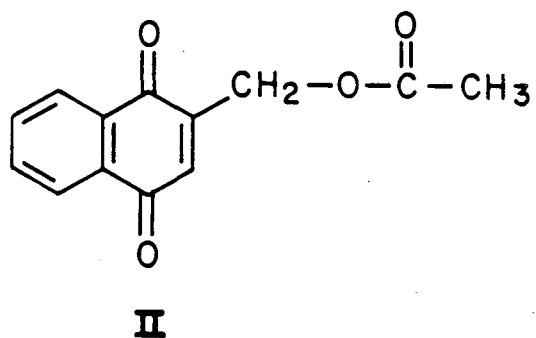
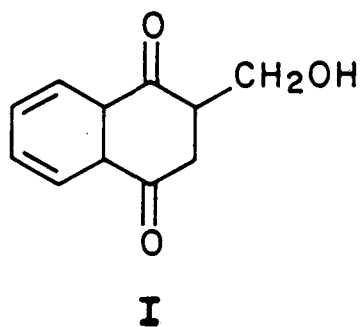
Inhibitions were performed by incubating the PSII particles at a Chl concentration of 5-10 $\mu\text{g Chl/ml}$ with the potential suicide quinone. The quinone to be tested was added to a final concentration of 3 μM . This corresponds to a quinone to P_{680} molar ratio of approximately 200/1. To test the proposal that it was electron transfer to the quinone which was responsible for inhibition, incubations were carried out in the dark and in the presence of weak illumination (3 mW cm^{-2}). Quinones were incubated for 5 min, after which DCBQ was added and O_2 evolution rates measured.

Five potential suicide quinones were tested for the ability to inhibit O_2 evolution in PSII particles. The compounds tested were: 2-hydroxymethyl-1,4-naphthoquinone (compound I); 2-acetoxymethyl-1,4-naphthoquinone (compound II); 2,3-bis(acetoxymethyl)-1,4-naphthoquinone (compound III); 2-bromomethyl-1,4-naphthoquinone (compound IV); and 2,3-dimethyl-5,6-bis(acetoxymethyl)-1,4-benzoquinone (compound V). The structures of these quinones are shown in Fig. IV-10. Compound III was provided by Dr. A. Sartorelli, Yale. All other compounds were synthesized by Stephen Isaacs (U. C. Berkeley).

b. Results

The quinones to be tested were added in acetone. The final volume of acetone in the O_2 evolution assay mixture was 0.05%. The effect of this concentration of acetone was assayed by incubating

Fig. IV-10. Structures of five potential suicide quinones tested for the ability to inhibit oxygen evolution.



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Fig. IV-10

PSII particles in exactly the manner in which quinone inhibition studies were done, except that no quinone was added in the acetone. After 5 min incubation in the dark, 0.05% acetone decreased the O_2 evolution rates to 96% of control rates. After 5 min incubation under illumination (3 mW cm^{-2}) 0.05% acetone decreased the O_2 evolution rates to 84% of control rates. All O_2 evolution rates measured in the presence of quinones were compared to the rates obtained with acetone alone. Rates for samples which were illuminated during incubation are expressed as percentages of the rates obtained after illumination in the presence of acetone alone. Rates for samples which were incubated in the dark are expressed as percentages of rates obtained after incubation in the dark in the presence of acetone alone.

The normalized rates of O_2 evolution after incubation with potential suicide quinones are given in Table IV-2. Incubation with the hydroxymethyl naphthoquinone (compound I) caused almost no inhibition, regardless of whether the incubation was in the dark or in the light. Incubation with either acetoxymethyl derivative of naphthoquinone (compounds II and III) caused substantial inhibition of O_2 evolution if the incubation was carried out under illumination, but no inhibition if the incubation was done in the dark. Interestingly, the acetoxymethyl derivative of benzoquinone (compound V) caused no inhibition if incubated in the dark, and actually stimulated O_2 evolution somewhat if incubated under illumination. The bromomethyl naphthoquinone inhibited O_2 evolution if incubated in the dark or the light. The inhibition was greater when illumination was present during incubation.

Table IV-2. Inhibition of O_2 evolution in PSII particles by a series of potential suicide quinones. See Fig. IV-10 for the structure of the quinone used. PSII particles were incubated for 5 min in the presence of quinone (quinone/ P_{680} molar ratio = 200) either in the dark or under illumination at 3 mW/cm^2 . O_2 evolution rates for illuminated samples are expressed as percentages of rates obtained after 5 min illumination in the presence of acetone alone. Rates for unilluminated samples are expressed as percentages of rates obtained after 5 min incubation in the dark in the presence of acetone alone.

Relative O₂ evolution rates obtained in the presence of potential suicide quinones

		dark incubation	light incubation
Compound	I	97	94
	II	101	55
	III	101	64
	IV	63	47
	V	101	114

Table IV-2

The nature of the inhibition caused by illumination in the presence of compound II was tested by using twice the concentration of DCBQ (electron acceptor) typically used in the O_2 evolution assays. Increasing the concentration of DCBQ had no effect on the rate of O_2 evolution. This indicates that the inhibition observed after illumination is not due to a reversible competition between compound II and DCBQ for a binding site on the PSII particle.

The proposed mechanism of action of suicide quinones involves reduction of the quinone, departure of a leaving group, and subsequent alkylation of the enzyme by the resultant reactive species. Incubation in the dark was used to test the importance of electron transport to inhibition. During incubation in the dark, no electrons are transferred from P_{680} to quinones located on the acceptor side of PSII. The two acetoxymethyl compounds tested (compounds II and III) showed no inhibition when incubated with PSII particles in the dark, yet caused substantial inhibition if illumination was present during the incubation. This is exactly the behavior expected if reduction of the quinone is a necessary prerequisite to inhibition. The bromomethyl derivative (compound IV), however, caused inhibition when incubated without illumination. This inhibition cannot be caused by electron flow from P_{680} , because P_{680} is not turning over in the dark. An increase in inhibition was seen when compound V was incubated in the light. The incremental inhibition seen under illumination may be due to reduction of the quinone by electrons from P_{680} .

The proposed mechanism of inhibition by suicide quinones predicts a dependence of inhibition on the reactivity of the

potential leaving group. Compounds I-IV are a series of naphthoquinone derivatives, with a range of leaving groups. The relative reactivities of the potential leaving groups is $\text{Br}^- >$ acetoxy \gg OH^- . By comparing the inhibitions obtained with the different naphthoquinone derivatives, and by looking at the dependence of inhibition on illumination, one can assess the importance of leaving group reactivity. The extent of inhibition by the different compounds is exactly as expected from the reactivities of the leaving groups. The hydroxymethyl derivative has a very unreactive leaving group (OH^-) and causes little if any inhibition. The acetoxy derivatives possess more reactive leaving groups, and cause substantial inhibition under conditions where one expects reduction of quinones (illumination of PSII particles). The bromomethyl compound possesses an even more reactive leaving group. In fact, Br^- is such a good leaving group that the bromomethyl derivative is a potential alkylating agent even in the absence of reduction. Inhibition in the dark (i.e. in the absence of quinone reduction) is expected if the unreduced bromomethyl compound is an alkylating agent. This is what was observed. Illumination of PSII particles in the presence of compound IV caused an increased inhibition. This is what one expects if the reduced bromomethyl naphthoquinone is a more reactive alkylating agent than the unreduced compound.

It should be noted that the discussion of inhibition by the series of naphthoquinone derivatives was made with the view that these compounds can accept electrons from PSII. This is reasonable, because a large number of quinone derivatives can accept electrons from

PSII, but I have not demonstrated that the quinones in question can in fact accept electrons from PSII. To demonstrate this, one must do extraction and reconstitution experiments similar to those described for bacterial RC's (see section 3). One of the quinones tested against PSII particles (compound III) was shown unequivocally to accept electrons from bacterial RC's. In light of the similarities between the acceptor sides of RC's and PSII (see Chap. I, part 2 and Wraight, 1982) it is reasonable to suggest that a potential suicide quinone which can accept electrons from RC's can also accept electrons from PSII particles. If the potential suicide quinones are in fact reduced by PSII, then the inhibition seen, and the dependence of inhibition on illumination, is understandable in terms of the reactivities of the potential leaving groups.

The acetoxymethyl benzoquinone derivative (compound V) tested gave results quite different from those of the naphthoquinone derivatives tested. Incubation in the dark caused no inhibition, while incubation in the light actually caused a slight stimulation of activity. The potential leaving group in compound V is identical to the potential leaving group in two active inhibitors (compounds II and III). The lack of inhibition has been observed three times, with two different preparations of PSII particles. In the work of Sartorelli and co-workers, benzoquinone derivatives were as potent as naphthoquinone derivatives as anti-tumor agents (Lin and Sartorelli, 1973 and Lin et al, 1973). At this time there is no definitive explanation for the lack of inhibition by compound V.

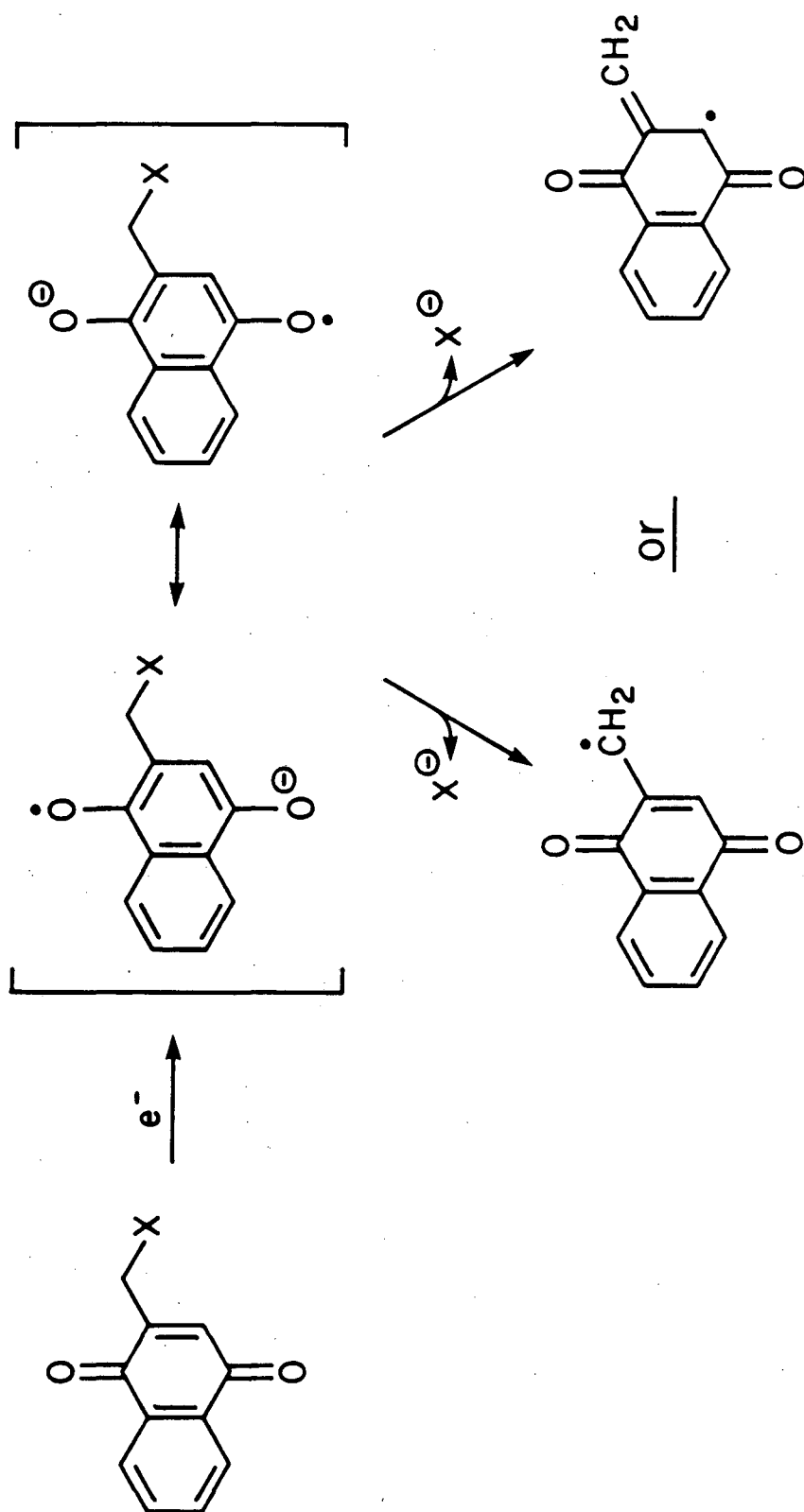
The lack of inhibition by compound V may be specific to the model system used in this study (PSII particles). It is interesting

to note that of the potential suicide quinones tested, compound V is the closest structural analogue to PQ, the native quinone acceptor of PSII. The proposed mechanism of inhibition by suicide quinones (Fig. IV-1) depicts decomposition of the dihydroquinone to form the quinone methide. An alternative mechanism (Fig. IV-11) is decomposition of the radical anion form of the suicide quinone to give a radical quinone and the anionic (i.e. unprotonated) form of the leaving group. If this mechanism prevails, any interactions which stabilize the semiquinone anion would be expected to decrease the efficiency of decomposition to the quinone radical and the anionic leaving group. The Q_b binding site probably stabilizes PQ in the radical anion form (Hearst and Sauer, 1984). Due to its structural similarity to PQ, compound V may be stabilized in the radical anion form, decreasing the probability of decomposition to form an alkylating intermediate. Compounds I-IV may bind at the Q_b site, but not in such a way as to experience any stabilization in the radical anion form. In this case, decomposition of the radical anion may proceed and result in inhibition of O_2 evolution.

5. CONCLUSIONS.

Extraction of RC's with 0.6% LDAO results in removal of 85% or more of the UQ-10 bound to the Q_b site, while removing none of the UQ-10 bound at the Q_a site. Extraction with 1.0% LDAO, 5 mM o-phenanthroline, results in removal of all UQ-10 from the Q_b site and 95% of the UQ-10 bound at the Q_a site. In both extraction procedures, the integrity of the RC is maintained, as shown by the preservation of the characteristic IR spectrum. Both procedures

Fig. IV-11. Alternative proposal for reductive activation of potential suicide quinones (contrast to Fig. IV-1 from Sartorelli).



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Fig. IV-11

result in reasonably high yields (85% for Q_b removal; 70% for removal of Q_a and Q_b) of the extracted RC's.

Incubation of UQ-10 depleted RC's with a 10 fold excess of UQ-10 results in full occupancy at the Q_a site. The recovery kinetics suggest an occupancy $\geq 90\%$, but these experiments should be repeated using the Aminco spectrophotometer. Kinetic data for times less than one second will allow an accurate determination of the Q_b occupancy. The ability to re-occupy the quinone binding sites indicates that the extraction procedure does not result in an irreversible degradation of the binding sites.

2,3-bis(acetoxymethyl)-1,4-naphthoquinone is capable of occupying the Q_a site of RC's and accepting electrons from the primary donor. Reconstitution of UQ-depleted RC's with a 100 fold excess of the naphthoquinone gave $\geq 94\%$ occupancy at the Q_a site. The kinetic data suggested that 60% or more of the Q_b site was also occupied, but this is not a definitive result.

A series of potential suicide quinones were tested for their ability to inhibit O_2 evolution in PSII particles. Acetoxymethyl and bromomethyl derivatives of naphthoquinone inhibit O_2 evolution significantly. Inhibition by the bromomethyl derivative occurs with or without illumination, whereas inhibition by the acetoxymethyl derivatives is strictly dependent on illumination during incubation. The inhibition by one of the acetoxymethyl derivatives is not reversible by higher concentrations of DCBQ. A hydroxymethyl derivative of naphthoquinone fails to inhibit O_2 evolution after incubation in the dark or the light. An acetoxymethyl derivative of benzoquinone also is uninhibitory, either after incubation in the

dark or the light. In the series of naphthoquinone derivatives tested, the inhibition (or lack thereof), and the dependence of inhibition on illumination, correlates with the relative reactivities of the potential leaving groups.

The two acetoxymethyl derivatives of naphthoquinone appear to be the most promising candidates as suicide quinones. These compounds cause significant inhibition of O_2 evolution. The inhibition is strictly dependent upon illumination, suggesting that reduction by P_{680} is necessary to activate these inhibitors. The inhibition by compound II is not reversible with higher concentration of electron acceptor. Based on these results, the two acetoxymethyl derivatives are presently being prepared in tritiated form. This will permit testing of the idea that inhibition of O_2 evolution is accompanied by alkylation of the PSII particle by the suicide quinone.

CHAPTER V SUMMARY AND PROSPECTS FOR FUTURE WORK

a. Introduction

Photosynthetic reaction centers convert the energy of a photon into chemical potential. This conversion is accomplished rapidly and with a very high quantum yield. A main goal of photosynthetic research is to understand the structural basis for the efficient energy conversion accomplished by the reaction center. Particularly interesting are interactions between the cofactors (pigments and electron acceptors) and polypeptides of the reaction center. By themselves in solution, the cofactors are capable of absorbing photons, and undergoing oxidation-reduction reactions, but efficient energy conversion does not occur. Stabilized charge separation requires the complexation of the cofactors with the protein portion of the reaction center. Conversion to biologically useful forms of energy requires the asymmetric incorporation of the reaction center complex into a membrane. Presumably, interactions between the protein and cofactors are important for maintaining the cofactors in the proper orientation with respect to each other. Interactions with the protein are probably also important in altering the light-absorbing and electron transfer properties of the cofactors. The efficient energy conversion which takes place in a reaction center is therefore a result of the particular arrangement of the cofactors and polypeptides. A major goal of photosynthetic research is to determine the arrangement of the components of a reaction center, and to deduce from this structure some of the elements responsible for the catalytic efficiency demonstrated by the reaction center.

Bacterial reaction centers (RC's) have been extremely useful for structural investigations. This is primarily due to our ability to isolate large amounts of very pure RC's. The isolated complex is fairly small (100 kD) and has a well-defined composition. Another advantage of bacterial systems is the availability of genetic techniques. Mutational analysis allows one to investigate particular complexes in the photosynthetic apparatus, and to address the interactions between different complexes. Recently, the application of genetic techniques has provided the amino acid sequences of the RC subunits from Rps. capsulata (Youvan et al, 1984 a,b). This major advance is a prerequisite to a complete understanding of the structural basis of catalysis by the RC. Understanding the RC also requires the application of spectroscopic techniques for structure determination. Spectroscopic investigations have focussed primarily on the cofactors of the RC. Recently, spectroscopic studies have been extended to an X-ray diffraction analysis of single crystals of RC's from Rps. viridis (Michel, 1982). Knowledge of the three-dimensional structure of the RC is another prerequisite to understanding energy conversion in photosynthetic organisms. The bacterial reaction center, then, is a system in which biochemical, genetic, and spectroscopic techniques can all be brought to bear on the question of energy conversion.

b. SUMMARY OF THESIS WORK.

The work described in this thesis concerns the RC's from Rps. capsulata and Rps. sphaeroides. A new technique is described for the isolation of active reaction centers. The polypeptide subunits

of the RC were isolated by preparative electrophoresis. The amino-terminal sequences for each subunit are reported. Techniques were developed for fragmentation of the RC subunits. The quinones of RC's were extracted. The quinone binding sites were reconstituted with ubiquinone and a non-physiological quinone. A new class of potential suicide inhibitors of electron transport were assayed for their ability to inhibit O_2 evolution in PSII particles.

Active RC's were isolated by affinity chromatography on equine cyt c. Affinity chromatography allows isolation of RC's without the use of precipitating agents at any step in the purification. This may be important for the growth of single crystals of RC's. The procedure involves removing loosely bound proteins from the chromatophore membranes, solubilization of the RC's with detergent, and adsorption of the RC's to the affinity column. RC's are eluted from the cyt c column with increased ionic strength. In its final form, i.e. as applied to isolation of RC's from Rps. capsulata, strain KZR8A1, the technique involves the use of a non-linear salt gradient to separate RC's from other proteins which bind to the cyt c column. With this adaptation, the affinity chromatography technique yields RC's which are as pure, with respect to contamination by other polypeptides, as any prepared by the other published procedures for isolation of RC's.

The integrity of the RC's isolated by affinity chromatography was tested by several means. The RC's from Rps. sphaeroides possess the IR spectrum typically seen for this protein. This spectrum is a sensitive measure of interactions among the pigments and between the pigments and the protein. The RC's from Rps. capsulata, strain

KZR8A1, possess a similar IR spectrum, except that the position of the lowest-energy absorption band is blue-shifted 15 nm with respect to RC's from Rps. sphaeroides, strain R-26. Bleaching spectra recorded in intact chromatophores from strain KZR8A1 indicate that this blue-shifted spectrum is a fundamental property of the RC, and not a result of denaturation during isolation. RC's isolated by affinity chromatography are capable of undergoing charge-separation, as demonstrated by light-induced changes in the absorption spectrum. For RC's from strain R-26, a light-induced EPR spectrum was also recorded. The line-shape and decay kinetics of this signal are very similar to previous results, indicating little disruption of electron donor-electron acceptor interactions during isolation.

The three subunits of RC's from Rps. capsulata, strain KZR8A1, were isolated by preparative electrophoresis and electroelution. The isolated subunits were submitted for a determination of the amino-terminal sequences (7-16 amino acids) in a gas-phase sequenator. In each case, the first residue of the protein was not assignable. Exact correspondence was seen between the residues assigned by Edman degradation and the sequence deduced from the DNA.

The extent of processing at the amino-terminus of each subunit was deduced by comparing the polypeptide sequences to the sequences deduced from the genes. The M and L subunits are processed so as to remove the amino-terminal Met. The amino-terminal residue in the mature M and L polypeptides is Ala. The H subunit is not processed at the amino-terminus. None of the subunits is processed at the amino-terminus more extensively than to remove one amino acid.

The RC subunits were dissolved in SDS (+DTT for the L subunit)

and fragmented. H and M each contain one Asp-Pro peptidyl bond. This bond is very susceptible to acid cleavage. H was cleaved into two fragments by incubation in 90% formic acid. Both fragments were isolated by preparative electrophoresis. The apparent molecular weights of the fragments are very close to the molecular weights expected from the position of the Asp-Pro linkage in the deduced amino acid sequence. This provides an important confirmation of the deduced amino acid sequence for the H subunit.

The M subunit was digested with 80% formic acid. Only one fragment could be detected. This fragment was isolated by preparative electrophoresis. The apparent molecular weight was 2 kD less than the molecular weight expected for the large fragment generated by acid cleavage. A small fragment (9 kD) was also expected from acid cleavage, but was not detected.

The L subunit was digested with S. aureus V8 protease, under conditions such that the enzyme cleaved only after Glu residues. One digestion product with an apparent molecular weight of 23.2 kD was generated. The deduced amino acid sequence for the L subunit contains two potential sites for cleavage by the protease, both of which would yield fragments of approximately 23.2 kD molecular weight. It is not possible yet to distinguish which of these two sites was cleaved. Cleavage at either site would generate a small (8 kD) fragment. This fragment was not detected.

RC's contain two quinones as electron acceptors. The two quinone binding sites can be distinguished on the basis of the conditions required to extract the quinone. RC's extracted with 0.6% LDAO still contain one quinone, as shown by the ability to photo-

oxidize the primary donor. RC's extracted with 1.0% LDAO, 5 mM o-phenanthroline contain only 0.06 quinone/RC, as assayed by the (in)ability to oxidize P_{850} .

RC's essentially free of quinone were reconstituted with ubiquinone. Reconstitution of RC's with a 10 fold excess of ubiquinone gives full occupancy at the Q_a site, and nearly full occupancy at the Q_b site. RC's devoid of quinone were also reconstituted with a 100 fold excess of 2,3-bis(acetoxymethyl)-1,4-naphthoquinone, a potential suicide quinone. This compound fully restored the ability to photo-oxidize P_{850} . This indicates that the potential suicide quinone can occupy the Q_a site and accept electrons from P_{850} .

A series of potential suicide quinones was tested for their ability to inhibit O_2 evolution in PSII particles. Several derivatives of naphthoquinone were tested. Naphthoquinone derivatives containing one or two acetoxy substituents as potential leaving groups inhibit O_2 evolution if incubated with PSII particles under dim illumination, but are completely without effect if incubated in the dark. Inhibition by one of these derivatives is not reversed by increasing the concentration of electron acceptor in the O_2 evolution assay. (The reversibility of inhibition by the other acetoxy derivative was not tested.) A naphthoquinone derivative containing bromide as a potential leaving group inhibited O_2 evolution if incubated in the dark or under illumination. The inhibition was greater if the incubation was done under illumination. A hydroxy derivative of naphthoquinone was not inhibitory if incubated in the dark and only slightly inhibitory in

the light. Interestingly, an acetoxy derivative of benzoquinone was not inhibitory if incubated in the dark and actually slightly stimulatory if incubated in the light. The results obtained for the naphthoquinone derivatives were explained by assuming that these compounds are reduced by electrons from P_{680} when incubated in the light. (One derivative was shown to accept electrons from P_{850} in bacterial RC's.) The inhibition by the various derivatives was explained in terms of the relative reactivities of the potential leaving groups.

c. SPECULATIONS AND FUTURE PROSPECTS

The discussion at the beginning of this chapter emphasized that interactions between the cofactor and protein portions of RC's may be important determinants of the catalytic efficiency of RC's. To probe this idea further, one must know which portions of the protein interact with which cofactors. One approach to this problem is to prepare reactive analogues to a particular cofactor, and attempt to form a covalent bond between the cofactor analogue and the protein. Particularly useful are suicide analogues, in which catalysis by the RC is responsible for activation of the analogue. With suicide cofactor derivatives, covalent attachment between the cofactor and the protein should occur only in those instances where the cofactor analogue is bound at a catalytically competent site. The potential suicide quinones discussed in Chap. III were studied with the idea of finding a suitable reagent for labelling quinone binding sites. The two acetoxymethyl derivatives of naphthoquinone appear to be the most suitable compounds for use as suicide labelling reagents. These

two compounds are presently being prepared in tritiated form. With tritiated quinones it will be possible to address the question of covalent attachment between the cofactor analogue and the protein portion of the RC. After illumination of RC's or PSII particles, noncovalently bound quinones will be separated from the protein, either by ion exchange chromatography or gel electrophoresis. Covalently bound quinones can then be detected by scintillation counting or autoradiography. Gel electrophoresis followed by autoradiography will be particularly useful, because it will allow assignment of the polypeptide(s) labelled by the suicide analogue.

Fragmentation of RC polypeptides can be a useful complement to suicide labelling reactions. Ultimately, we wish to know which amino acids are involved in binding cofactors. If one is successful in forming a covalent bond between a cofactor analogue and the protein, fragmentation techniques can be applied to assign the site of attachment to a small region of the protein. This approach is currently being followed to map the site of attachment between azidoatrazine (a quinone antagonist) and the Q_b binding protein of chloroplasts (Wolber and Steinback, 1984). Preliminary indications are that the site of attachment is very close to, or contained within, the sequence region of the Q_b protein which is homologous to the L subunit of RC's. Hopefully, the technique of suicide labelling and fragmentation can be used to map the positions of the quinone binding sites in RC's. The fragmentation techniques presented for RC subunits (Chap. III) must be extended significantly to generate small fragments. Hopefully, suicide labelling will also

be applicable to mapping quinone binding sites on other quinone reductases, including PSII preparations from chloroplasts and cyanobacteria.

Another approach to studying structural aspects of RC's is X-ray crystallography. This requires three dimensional crystals of RC's. Several research groups have now been able to obtain crystals of RC's from Rps. viridis and Rps. sphaeroides. To date, the most detailed analysis has been done in Michel's laboratory. The preliminary information on the RC structure indicates a very interesting structure. At the resolution obtained so far, the electron donors and acceptors of the RC possess a fairly high degree of symmetry. At this point the path of electron flow within the RC is not obvious. Seemingly, there are two almost equivalent sets of acceptors (BChl + BPhe) on either side of the primary donor. A challenge for the future is to coordinate the X-ray structure with the wealth of spectroscopic information available concerning the relative orientations of the donors and acceptors. Also, it will be very interesting to see how the RC is inserted into the photosynthetic membrane. It is the non-random orientation of the RC within the membrane that allows primary charge separation to be coupled to the establishment of an electric field and a proton gradient. A major advance in structural studies of photosynthetic complexes would be the successful crystallization of a reaction center from cyanobacteria or higher plants. This would allow us to discern some of the general requirements for efficient photosynthetic energy conversion.

Theoretical studies predict that coupling between vibronic and

electronic excited states of BChl may be important for excitation transfer between pigments. Likewise, coupling between vibronic and electronic excited states of BChl, or between vibronic and electronic excited states of BPhe or quinone, may be important in determining the rates of electron transfer reactions between donors and acceptors. For these reasons it is important to study the vibrational modes of the BChl's, BPhe's, and quinones when bound to the protein. Resonance Raman studies are probably the most direct method for studying the vibrational modes of pigments. It will be particularly interesting to compare the resonance Raman spectra of the cofactors in solution and in the RC complex. Any changes in vibrational modes may indicate a role for the protein portion of the RC in modifying the solution behavior of the cofactors so as to produce efficient energy conversion.

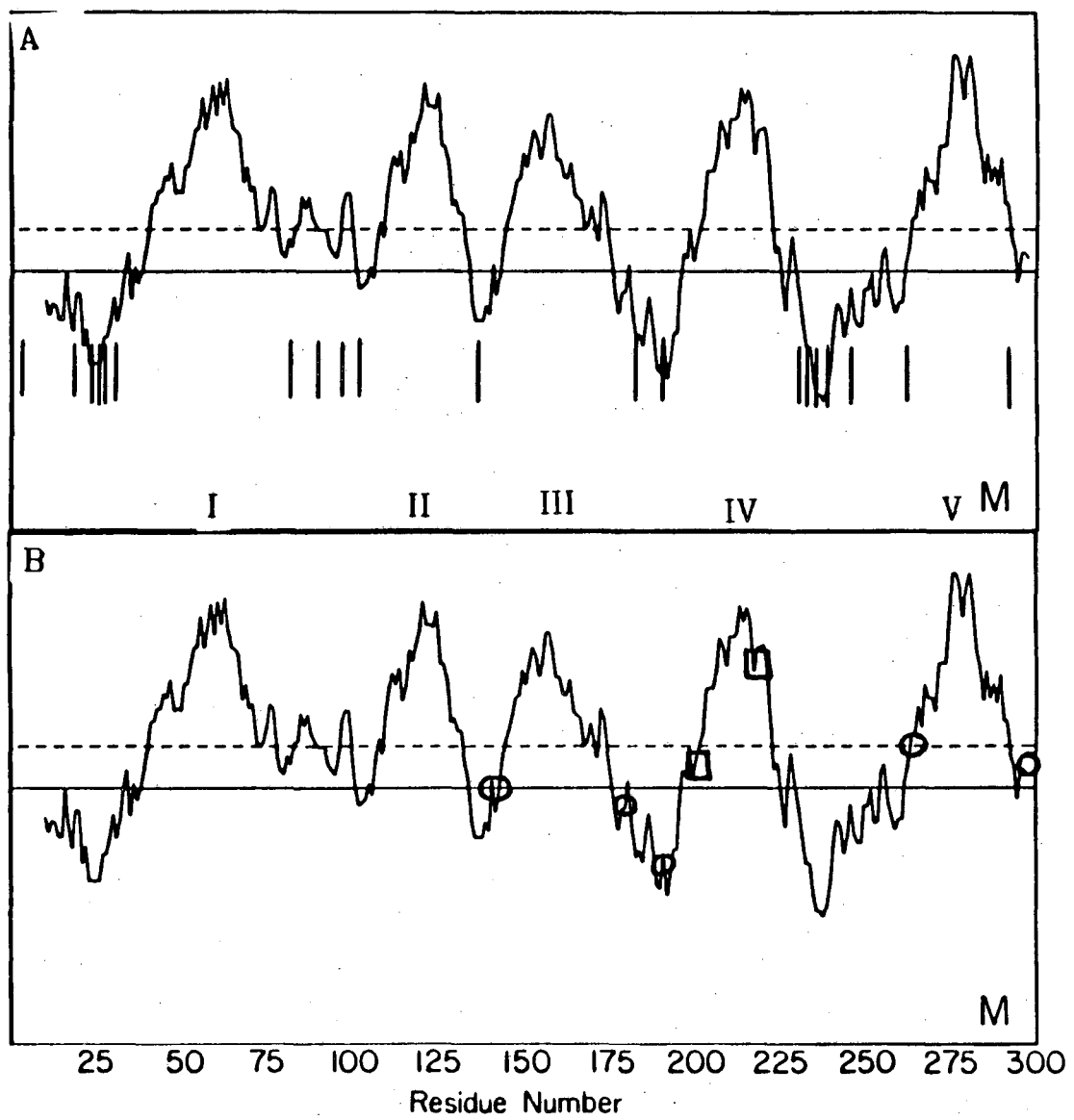
Another approach to looking at protein-cofactor interactions within the RC is wild speculation. This serves the dual purpose of suggesting future experiments and providing amusement for idle graduate students and professors. The recently determined amino acid sequence of the RC polypeptides provides material for interesting speculation. I have paid particular attention to the distribution of His, Glu, and Asp residues within the sequences of the M and L subunit. The rationalization for concentrating on His residues is that in the known structure of a water-soluble Chl protein, 6 of 7 Chls are ligated by His residues. The binding of cyt c_2 to the RC involves acidic residues. The reason for looking at the M and L subunits is that these polypeptides are known to bind all of the pigments of the RC as well as cyt c_2 .

My speculations about RC structure developed from examining the hydropathy plots for the M and L subunits (from Youvan et al, 1984b). They also rely heavily on the preliminary structure of RC's from Rps. viridis (Michel et al, 1984). It has never been demonstrated that hydropathy plots are valid descriptions of the folding patterns for a wide variety of membrane proteins. Thus it is imperative to view predictions based on hydropathy plots with a healthy skepticism. Nevertheless, I will offer some interesting, and testable, ideas concerning the arrangement of cofactors within the RC and the orientation of the RC protein within the chromatophore membrane.

Fig. V-1A shows the hydropathy plot for the M subunit. The positions of the twenty acidic residues (Asp and Glu) are shown with hash-marks. As expected, the acidic residues lie within the regions of the protein which are predicted to lie outside the membrane. If one assumes that each peak in Fig. V-1A represents a membrane-traversing segment, one can determine whether the distribution of acidic residues is random with respect to the membrane. In fact it is not. The amino-terminus of the M subunit appears to lie outside in the membrane. For now, call this side A. After the amino-terminus, the next hydrophilic section will lie on the B side of the membrane. The next hydrophilic section will be back on the A side, and so on. In all, 13 of the acidic residues lie on side A of the membrane, while 7 of the acidic residues lie on side B. Because cyt c_2 binds to acidic residues, and cyt c_2 is localized on the periplasmic side of the membrane, it is more likely that side A of the membrane is the periplasmic side. This model predicts that

Fig. V-1. Hydropathy plot for the M subunit of reaction centers from Rps. capsulata (from Youvan et al, 1984b). A) acidic residues (Asp and Glu) are shown with hash marks. B) His residues are marked. Squares indicate His residues which may be involved in quinone binding (Hearst and Sauer, 1984). Other His residues may be involved in BChl binding. Proposed membrane-spanning regions are numbered I-V.

Fig. V-1



the amino-terminus of the M subunit is on the periplasmic side of the membrane. The model also predicts that modification of the acidic residues clustered near residue 25, or modification of the acidic residues clustered near residue 230, would alter the binding of cyt c to the RC. Modifications have been done on the acidic residues, and shown to alter cyt c binding (Okamura and Feher, 1983). As yet, the positions of the modified residues have not been determined.

Fig. V-1B shows the hydropathy plot of the M subunit, with the 7 His residues marked with circles and squares. It has been proposed that His 201 and His 218 are involved in quinone binding (Hearst and Sauer, 1984). For the sake of making even more predictions, this proposal is adopted here. To suggest possible interactions with quinones, His 210 and His 218 are marked with squares. The other His residues are marked with circles. These residues are possible BChl binding sites. His 191 is in a hydrophilic stretch of amino acids, making it an unlikely candidate for a BChl binding site. This leaves His residues 144, 181, 265, and 300 as potential BChl binding sites. The preliminary X-ray structure of RC's from Rps. viridis suggests that all four BChl's are in a fairly thin transverse section of the membrane. This means that the His residues involved in BChl binding should all be within a thin section of the membrane as well. The primary donor is most likely on the periplasmic side of the membrane, because it accepts electrons from cyt c_2 . Based on this, I predict that His residues involved in BChl binding will be found on the periplasmic side of the membrane. It was already proposed that the hydrophilic segment

between regions IV and V lies on the periplasmic side of the membrane. This permits a prediction as to which His residues are involved in binding BChl. According to my model of the RC orientation within the chromatophore membrane, His residues 144 and 265 lie near the periplasmic side of the membrane, while His residues 181 and 300 lie nearer the cytoplasmic side of the membrane. Therefore, I predict that His residues 144 and 265 are involved in BChl binding.

The same ideas can be applied to the L subunit. Fig. V-2A and V-2B show hydropathy plots of the L subunit, with the acidic residues and the His residues marked. As in the M subunit, His residues 174 and 191 are marked with squares, to suggest possible quinone binding sites. The distribution of acidic residues is not so asymmetric as in the M subunit (8 acidic residues are on one side of the membrane, 6 are on the other side). Still, the cluster of acidic residues near residue 210 is the most prominent cluster of acidic residues in the sequence. Based on this, and on analogy to the M subunit, I propose that the hydrophilic stretch of residues between segment IV and V is on the periplasmic side of the membrane and interacts with cyt c_2 . His residues 117 and 231 probably lie on the same side of the membrane as the proposed cyt c binding site. Therefore, I suggest that His residues 117 and 231 are involved in BChl binding.

In all, I have suggested 4 His residues which might be involved in BChl binding. The number of His residues was chosen to conform to the number of BChls in the RC (4). If these residues are actually involved in binding BChl, at least two structures can be

Fig. V-2. Hydropathy plots for the L subunit of reaction centers from Rps. capsulata (from Youvan et al, 1984b). A) acidic residues (Asp and Glu) are shown with hash marks. B) His residues are marked. Squares indicate His residues which may be involved in quinone binding (Hearst and Sauer, 1984). Other His residues may be involved in BChl binding. Proposed membrane-spanning regions are numbered I-V.

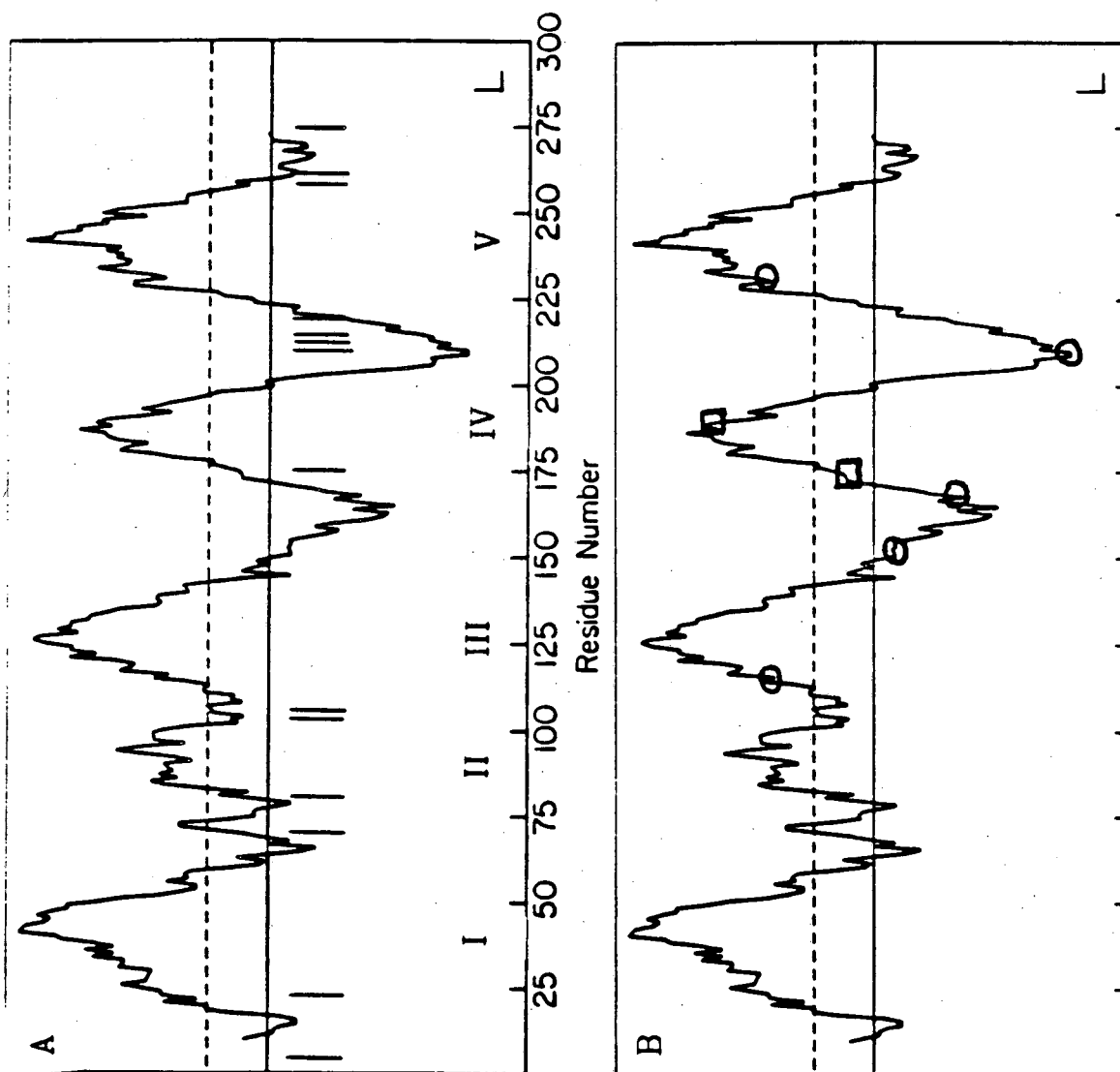


Fig. V-2

envisioned. The X-ray structure of RC's from Rps. viridis shows two BChls whose porphyrin rings are nearly touching. Two other BChls lie on either side of the two close BChls. A possible arrangement is one in which the two closely spaced BChls (perhaps the special pair) are bound to one subunit, while the two outer-lying BChls are bound to the other subunit. Another possibility is that each subunit contributes one His to bind a BChl of the closely spaced pair, and each subunit contributes one His to bind one of the outer-lying BChls. At present I prefer the latter model. Having each subunit folded in such a way as to bind one side of the paired BChls may lead to the seemingly symmetric structure seen in the X-ray structure. Also, sequence homologies between the M and L subunit suggest that they arise from a common precursor. One can imagine an ancestral RC which contained only one BChl as a primary donor, and one other BChl as a very early electron acceptor. Gene duplication followed by divergence of the sequences would lead to two slightly different RC structures. Dimerization of these structures could have led to a RC in which the primary donor was a BChl dimer. This might also explain the structure of electron acceptors seen in the Rps. viridis structure, in which there seem to be two almost equivalent pathways for electrons to follow.

The proposed folding pattern was developed by assuming that the putative quinone binding sites (His residues 174 and 191 in the L subunit, and His residues 201 and 218 in the M subunit) are in fact correct. Because of this assumption, the suggested quinone binding sites should be on the opposite side of the membrane from the proposed primary donor site. This is the case. If quinones are

bound in such a fashion that they interact with both His residues of the proposed sites, then, according to the proposed folding pattern, each quinone will reach from the middle of the membrane towards the cytoplasmic side. With quinones in this location and orientation, electron transfer would generate an electric field across the membrane, as in fact it does.

The tests of my speculations will be two-fold. A higher resolution structure will determine if His residues are involved in BChl binding. If this is the case, it will be interesting to see whether the residues emphasized here on the basis of predicted folding turn out to be involved in BChl binding. Prior to a higher resolution structure, chemical modification studies can be attempted to assess the importance of particular residues. Capaldi and coworkers have modified specific acidic residues in cyt oxidase to determine which residues are involved in cyt c binding (Millet et al, 1983). This same approach may be successful in determining which acidic residues in RC's bind cyt c_2 . Chromatophores and spheroplasts have opposite orientations. It may be possible to modify acidic residues in chromatophores and spheroplasts to determine the orientation of RC's in the membrane. Diethylpyrocarbonate is capable of modifying His residues. This reaction may be useful in disrupting specific His:BChl interactions, and thus showing which His residues interact with which BChls. Also, each subunit contains His residues which seem to be in hydrophilic regions of the protein (see Figs. IV-1B and IV-2B). These His residues may be susceptible to labelling with diethylpyrocarbonate while the RC is embedded in a membrane or lipid bilayer. The proposed folding pattern for the L subunit predicts that

His 212 lies on the periplasmic side of the membrane. It may be possible to label chromatophores and spheroplasts with diethylpyrocarbonate to test this idea. Also, binding of cyt c_2 may protect this His residue, since it lies in the heart of a cluster of acidic residues. Hopefully, labeling studies such as those proposed will help determine the orientation of the RC within the chromatophore membrane.

Another area where advances will surely be made is the application of the techniques of molecular genetics to studying RC's. This approach has already given us the sequence of the RC polypeptides. A particularly exciting possibility is the use of site-directed mutagenesis to probe the importance of specific amino acids in the catalytic cycle of the RC. The genes for the RC polypeptides have been cloned into the mutable plasmid pBR322. The constructed plasmids contain antibiotic resistance markers applicable to selection in E. coli or Rps. capsulata. Also available are plasmids to mediate the conjugation of a mutant pBR322 derivative from E. coli into Rps. capsulata. (To date, direct transformation of Rps. capsulata has not been possible, although recently a transformation system for Rps. sphaeroides was developed.) The availability of the cloned genes and the conjugation-mediating plasmids means that one can mutagenize a Rps. capsulata gene in E. coli and then incorporate the mutated gene into the Rps. capsulata genome. This has already been accomplished using transposon-mutagenized genes (Zsebo and Hearst, 1984). By using synthesized oligonucleotides one can generate any base-pair change desired. This means it is possible to change a given amino acid

codon in a gene into another codon. Expression and characterization of the mutant protein than allows one to begin assessing the importance of the altered amino acid in the function of the protein.

In RC's, one way to proceed along these lines would be to mutagenize sites which are predicted to interact with the cofactors of the RC. For example, the sequences which are conserved between M, L, and the Q_b protein could be mutagenized to investigate the importance of these sequences to quinone binding. Electron transfer from Q_a to Q_b has been shown to be dependent on pH, with an apparent pK of 9-10. In both the L and M subunits, the putative quinone binding regions contain Tyr residues. This residue could be altered to Phe by a change in one base. A mutant RC containing Phe in place of Tyr may possess interesting electron transfer kinetics whose pH dependence could be investigated. Another possibility, of course, is that the mutant RC may be completely non-functional. Such a result cannot be interpreted in terms of the specific role of a particular residue. Another possibility for site directed mutagenesis is to try to generate a mutant RC which has lowered susceptibility to herbicides. Herbicide resistant mutants from Rps. sphaeroides, Chlamydomonas, and higher plants have been isolated by selection of populations subjected to the continuous presence of herbicides. It has been shown that many of these mutants contain one altered amino acid residue. It would be interesting to begin selectively changing certain residues in capsulata RC's and then looking for altered herbicide susceptibility. Finally, one could investigate the importance of His residues in BChl binding by altering these residues, either in RC's or light-harvesting

pigments. Again, these mutations may result in fairly uninteresting proteins which are completely devoid of function.

It is important to remember that the goal of site-directed mutagenesis is to define the role of specific residues in the catalysis or structure of the protein. To properly interpret the results, one must know what effect the mutation has on protein structure and cofactor binding. One approach is to look for gross conformational changes in the protein, using circular dichroism or infra-red spectroscopies. These experiments give an indication of only the overall conformation of the protein. They may be insensitive to small changes in the protein structure, but it may be just these small changes which are important for interpreting a mutagenesis experiment. The best approach to study the effects of site-directed mutagenesis is to determine the three-dimensional structures of the wild-type protein and any interesting mutant proteins. A coordinated approach of generating specific mutants, investigating their spectroscopic properties, and determining the structural basis for any changes observed would go a very long way towards elucidating the important structural determinants of RC function.

Several techniques for studying RC's have been described in this thesis or suggested in this prospectus for future work. These same techniques can be applied to other components of the bacterial photosynthetic apparatus. A very interesting complex is the ubiquinol-cyt c oxidoreductase (bc_1 complex). This complex is involved in aerobic and photosynthetic electron transport. Related complexes are found widely dispersed in nature. It would be interesting to study the structural determinants of this protein's

function, in a manner analogous to those described for RC's. Another intriguing protein is a 44 kD heme-containing protein (see Zsebo, 1984). Transposon insertions affecting expression of this protein also affect expression of the RC polypeptides. Preliminary results (Trachiotis and Worland, unpublished observations) indicate that this heme protein may be involved in transferring electrons from substrate pools into the photosynthetic electron transport chain.

So far, I have concentrated on complexes found in photosynthetic bacteria. The same questions can be asked about the complexes found in higher photosynthetic organisms. In the past, a drawback to studying RC's from higher organisms was the difficulty of isolating small, well-defined reaction center complexes. Preparative electrophoresis has been used recently to partially surmount this problem. Reaction center preparations from PSI and PSII have been isolated in this fashion. A drawback to the electrophoretic techniques is the very low amounts of material which can be isolated. Development of conventional (i.e. non-electrophoretic) chromatographic techniques which yield reaction center preparations as small and well-defined as those isolated by electrophoresis would be a major accomplishment. The advantage of chromatographic techniques is that, once developed, they can usually be scaled up to give yields in the 10-100 mg range.

Another problem hampering the study of photosynthetic complexes from higher organisms is the genetic complexity encountered. In higher plants, photosynthesis requires the coordinated expression of the chloroplast and nuclear genomes. This makes the analysis of

mutations considerably more difficult than in bacteria. The techniques for transformation of plants are just now being developed. A fuller understanding of plant genetics will help elucidate the mechanisms whereby the expression of the photosynthetic apparatus is controlled. This understanding will also hopefully provide us with the genetic techniques which are so useful in investigating bacterial photosynthesis.

Cyanobacteria seem to be a logical step on the path of expanding our knowledge to higher photosynthetic organisms. Progress is being made on the isolation of complexes from these organisms. Genetic transformation systems have also been worked out, opening up many possibilities for experiments similar to those performed in Rhodospseudomonas.

The experiments I have been describing have primarily focused on understanding the structural basis of photosynthetic energy conversion. Equally interesting is an understanding of how organisms regulate the expression and assembly of such a complex structure as the photosynthetic apparatus. Rhodospseudomonas is an ideal system for addressing many of these questions. By controlling the growth conditions, one can control the type of energy metabolism utilized by the cell. Abrupt changes in growth conditions then allow one to study the response of the organism to environmental stimuli. The response must include the expression of new genes appropriate for the new growth conditions, as well as assembly of a complex apparatus. Hopefully, investigations in Rhodospseudomonas will shed light on the mechanisms of these crucial biological responses in other organisms.

Photosynthetic organisms have evolved over billions of years. A crucial determinant in this evolution has been the interaction between light and the photosynthetic apparatus. Because this interaction is at the core of the biological function, photosynthetic organisms are an ideal biological system for spectroscopic investigations. Undoubtedly, photosynthetic research will continue to benefit from the overlap between biological and physical investigations. In fact, photosynthetic research will continue to serve as a test ground wherein the most fruitful methods for a combined biological and physical approach to problems will be developed.

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