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IN BACTERIORHODOPSIN

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INTRODUCTION

Light energy conversion by bacteriorhodopsin involves vectorial translocation of a proton across the purple membrane of Halobacterium halobium. Following photon activation of the retinal chromophore, a photoreaction cycle commences. During the first 30-50 μsec of this photocycle, the light-adapted 570 nm chromophore is converted into the M_{412} transient species, whose Schiff base nitrogen has been found by resonance Raman spectroscopy (1) to be a deprotonated species. Reprotonation of M_{412} occurs during the 5-7 msec required for completion of the photocycle. These results suggest that deprotonation and reprotonation of the retinal Schiff base are essential for proton translocation across the membrane.

Since the primary sequence of bacteriorhodopsin has been established by Ovchinnikov et al. (2) and Gerber et al. (3), it is possible now to gain more information on the role of specific amino acid residues in proton translocation. Chemical modification of amino acids is a particularly promising approach to the problem because bacteriorhodopsin is the only protein component in the purple membrane and because the activity of these preparations is stable (4,5).

We report here the effect of treating purple membranes with two reagents which modify arginine residues and three carbodiimide reagents which modify carboxylic acid groups. In both cases, proton release in the early stages of the photocycle is slightly affected, but marked inhibition of the reprotonation phase occurs, leading to large increases in the amount of the M_{412} species seen in the photostationary state. The results suggest an essential role of the positively charged guanidinium group of arginine and negatively charged carboxyl containing

residues of bacteriorhodopsin in the decay of the M_{412} species of the photoreaction cycle at the inner surface of the purple membrane, where proton uptake from the intracellular space occurs. These two types of amino acids may be acting jointly to maintain structure or interacting via their opposite charges to catalyze proton translocation by bacteriorhodopsin.

MATERIAL AND METHODS

Purple membranes were purified by a modification of (6) from Halobacterium halobium strain S-9 cells grown according to (7). Purple membranes (0.3 - 0.9 mg bacteriorhodopsin/ml suspension) were treated with 2,3-butanedione (Aldrich Chemical Co.) in 50 mM borate buffer, pH 8.2 with 133-200 mM of reagent for 3 hr at 37° C (8,9,10). Modification with phenylglyoxal (3-12 mM) was in 100 mM bicarbonate buffer at pH 8, 25° C with 0.4 - 0.9 mg bacteriorhodopsin/ml purple membrane suspension (11,12). Quantitation of the extent of reaction of arginine and other amino acids was according to (13); the fluorescamine method (14) was also used for assaying free amino groups.

Purple membranes were treated with one of the following water soluble carbodiimides:

EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl, Sigma Chemical Co.

EAC: 1-ethyl-3-(4 azonia-4,4-dimethylpentyl) carbodiimide iodide (15)

CMC: 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate, Sigma Chemical Co.

in 100 mM MES buffer, pH 5.6, with 50 mM of reagent for 24 hours at 25° C. Carbodiimide solutions were prepared in 100 mM MES, pH 5.6 immediately prior to use. Glycine methyl ester hydrochloride was prepared in 100 mM

MES buffer and adjusted with NaOH to pH 5.6. Final concentration of glycine methyl ester in reaction mixture was 0.50 M. After reaction, purple membrane samples were diluted 10-fold with cold distilled water and centrifuged at 100,000 x g for 30 minutes; the supernatant was discarded and the procedure repeated twice and the samples resuspended in distilled water.

Spectrophotometric, fluorometric and flash-photolysis (16) assays were made at 0.1 - 0.2 mg protein per ml. Protein assays were either by the method of (17) with crystalline bovine serum albumin as standard or by 570 nm chromophore absorption (ε = 63,000 $M^{-1}cm^{-1}$) (18). Absorption spectra between 250-700 nm of light-adapted samples (740 mW/cm^2) were made in an Aminco DW-2 spectrophotometer. The percent of the 412 nm species in the photostationary state was also measured in this apparatus by side-illumination provided through a Corning 3-67 low wavelength cut-off filter having 50% transmission at 560 nm, 120 mW/cm^2, with the photomultiplier protected by a Baird Atomic 412 nm transmission interference filter. For flash photolysis studies, illumination was by a Phase-R dye laser with Rhodamine 575 (0.2 joules/flash, 150 ns flash risetime) at 25-26° C. The flash photolysis data was collected with a Biomation 1010 transient recorder. Tryptophan fluorescence of purple membranes was measured in a Perkin-Elmer MPF-44A fluorimeter at 285 nm excitation/335 nm emission. Light induced surface charge was measured as in (19) where the partitioning of an amphipathic spin probe between the aqueous medium and the purple membrane is used to sense changes in surface electrical charge.

RESULTS AND DISCUSSION

ARGININE MODIFICATION: After treatment of purple membranes with the

arginine specific reagents 2-3-butanedione and phenylglyoxal, their effect on the 570 nm chromophore, the photoreaction cycle, and upon other structural parameters was analyzed. Table I shows both reagents exert similar effects on bacteriorhodopsin. Treated purple membranes show little khange in 570 nm chromophore absorbance (with the reagent in place or removed by dialysis, in the case of butanedione), indicating little change in protein structure in the region of the chormophore has occurred.

The most marked effect of arginine modification was inhibition of the decay kinetics of the 412 nm transient intermediate, which shows a 35-fold inhibition, both with respect to the initial and secondary phases of the decay process. Although slight inhibition of the formation of the 412 nm species occurs, the net result following arginine modification, is a substantial increase in the amount of the 412 nm species in the photostationary state. Previous studies (19) have revealed that light induced surface charge changes in purple membranes are related to the amount of the 412 nm species, and in the butanedione-modified samples the surface potential change upon illumination was more negative than the controls (0.192 as compared to 0.103 moles negative charge/mole bacteriorhodopsin).

CARBOXYL MODIFICATION: Carbodiimides are highly selective for modification of carboxyl residues in proteins at slightly acidic pH (20). Absorbance of the 570 nm chromophore and tryptophan fluorescence is unchanged by carbodiimide treatment. However, Table II shows that after carbodiimide treatment, a marked inhibition of the decay of 412 nm intermediate is observed; no change in the risetime occurs. In the modified samples, a large increase in the photostationary state accompanies inhibition of

the 412 nm decay, as expected. For both the reagent alone and in the presence of glycine methyl ester, the biphasic character of the 412 nm decay becomes more pronounced, particularly for the EDC treated sample. Thus, the second phase of the 412 nm decay of this sample is approximately 50-fold slower than control values.

In general, carbodiimide-catalyzed amide formation results in an O-acyl isourea intermediate which in aqueous solution, either condenses with amines to yield corresponding amides, rearranges to form a more stable N-acylurea, or slowly hydrolyzes to regenerate the carboxyl group (21). Kinetic studies on model carbodiimide-carboxyl-nucleophile systems have shown that rearrangement can be rendered slow compared to nucleophile attack if the concentration of nucleophile is sufficiently high (22). Thus, when glycine methyl ester was present, carbodiimide treatment is expected to yield the amide adduct. Amino acid analysis performed on extensively washed and dialyzed samples after EDC/glycine methyl ester reaction showed twelve idditional glycine residues present over control values (36 vs. 24), hence, 60%, or 12 of the total 20 carboxyl groups had reacted. Under the mild conditions used, only the more accessible carboxyl groups will react. Since modification affects only the decay, and nst the rise of the 412 nm intermediate, it is clear that carboxyl groups are particularly important in proton uptake phase of the phstocycle.

A likely site for modification of carboxyl groups is the soluble C-terminal tail of bacteriorhodopsin. A study by Renthal et al. (23) using EDC, but under different reaction conditions at pH 5.5, indicates intermolecular cross-linking occurs between bacteriorhodopsin at this particular pH. It was shown that the C-terminal is responsible for oligomer formation. However, Abdulaev et al. (24) have demonstrated that there is no inhibitory effect on the ability of bacteriorhodopsin proteoliposomes

to generate a photopotential after proteolytic cleavage of the C-terminal 17 amino acid segment. Similar trypsin treated samples in our laboratory show no changes in photocycling characteristics (unpublished results). This implies that the five carboxyl groups on the C-terminal tail are unessential for proton translocation activity. Furthermore, in the case of EDC/glycine methyl ester modification, one can conclude that up to seven modified carboxyl groups remain on the protein which are important in the reprotonation process.

For the EDC treated samples, the reaction product has not yet been identified. Renthal et al., using EDC treatment under different reaction conditions (23), suggests that at pH 8.0, tyrosines are not involved in forming a O-aryl isourea product (25), rather the product is an intramolecular cross-link or an N-acyl urea. Both of these products would have a reduced number of negatively charged residues present on the protein. Obvious aggregation of the purple membrane suspension occurred during the reaction. Studies in our laboratory using the positively charged amphipathic spin label method (19) show a more positive surface charge for EDC treated purple membranes, consistent with the above possible reaction products.

STRUCTURE OF BACTERIORHODOPSIN: Using the information on the primary sequence of bacteriorhodopsin (26,27) a model of the tertiary structure of the molecule has been constructed (R.J. Mehlhorn collaboration, Figure 1). Of the fourteen positively charged amino acids (7 arginines and 7 lysines), and 20 (2) carboxyl containing amino acids, the highest proportion of negatively and positively charged groups are at the inner membrane interface. The hydrophobic amino acids are mainly in the protein interior as expected. The high charge density of negative and

positive charged groups at the inner membrane interface suggests that significant charged group separations may exist. Distance between the Schiff base of the chromophore and the cytoplasmic surface of the purple membrane is less than its distance to the extracellular surface. Nevertheless, reprotonation, which involves proton translocation from the cytoplasmic interior to the Schiff base, takes 100-fold more time than proton release at the extracellular surface. The longer time required for reprotonation could reflect the number of steps involved, the distance of proton movement, or existence of a structure having a high energy of activation which must be overcome in order for a proton to reach the Schiff base (28). It would appear from the chemical modification results that the latter may be the most significant.

There is a consensus (29) that following photon absorption by retinal in visual pigments and purple membranes, that electron redistribution and isomerization of the chromophore occurs. This results in a decrease in net positive charge of the Schiff base nitrogen, and a structural rearrangement leading to charge separation between groups in the protein as the primary energetic event. Knowledge of the precise structural arrangement of positively and negatively charged groups of amino acid residues in the vicinity of the chromophore are therefore of importance, as they may act as acceptors or donors for the translocated proton and for proton uptake. The essentiality of arginine and carboxyl residues as demonstrated by the chemical modification studies, suggests that either one or both of these groups may gain or lose a proton during the M_{412} reprotonation process. Generally, in enzymes where arginine residues have been found to be essential, these residues serve as the binding sites for carboxyl group containing substrates (30). In the present case, the carboxyl group of a nearby glutamate or aspartate residue may be the

substrate if it acts as a proton donor. The range over which the pK of carboxyl groups and of the guanidinium group of arginine in the protein may vary is unknown. Lewis et al. (28) has suggested that arginine residues may interact with the Schiff base nitrogen, and the present results tend to support this suggestion, but no conclusive evidence is available on the precise role of the guanidinium group of arginine, in terms of a direct interaction with the Schiff's base nitrogen in the proton transfer process. What is clear, is that the integrity of both the guanidinium group of arginine and carboxyl residues of glutamate or aspartate are essential in the proton uptake process which occurs from the cytoplasmic surface of the purple membrane to the Schiff base nitrogen of the chromophore. This may depend on one or more critically aligned salt bridges between the guanidinium and carboxyl groups of these amino acids and/or upon proton translocation from these groups.

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TABLE I

ARGININE MODIFICATION OF PURPLE MEMBRANES

Reagent	Sample	570 nm Chromophore	412 nm Photointermediate			
		Absorbance (%)	Rise ($t_{1/2}$ in μsec)	Phase of Decay[a] Initial ($t_{1/2}$ in msec)	Second	Photostationary Steady State Absorbance (%)
2,3-Butanedione	Control	100 (4)	45.4 (10)	0.86 (9)	5.4 (9)	100 (4)
	Treated	91.3 (4)	74 (11)	34.6 (10)	174 (10)	1922 (2)[b]
Phenylglyoxal	Control	100 (3)	35 (3)	0.78 (3)	5.03 (4)	100 (4)
	Treated	90 (4)	97.2 (6)	32 (6)	159 (6)	590 (2)

Parentheses indicate the number of samples averaged for calculation.

[a]Initial phase was calculated by subtracting the second slower phase extrapolated to the ordinate.

[b]Reagent removed.

TABLE II

CARBOXYLIC ACID GROUP MODIFICATION OF PURPLE MEMBRANES

Reagent	Sample	570 nm Chromophore Absorbance (%)	412 nm Photointermediate Rise ($t_{1/2}$ in μsec)	Phase of Decay ($t_{1/2}$ in msec) Initial	Second	Photostationary Steady State Absorbance (%)
EDC	Control	100	40	5.6	-	100
	Treated + glycine methyl ester	98.9	40	5.0	11.0	108
	Treated	85.7	40	50	280	527
EAC	Control	-	32	3.6	-	-
	Treated + glycine methyl ester	-	30	2.5	-	-
	Treated	-	28	5.0	20	-
CMC	Control	100	52	5.2	-	100
	Treated + glycine methyl ester	96.4	52	6.0	13.2	103
	Treated	83.3	60	20	170	417

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