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Structural Aspects of the Cytochrome b_6f Complex; Structure of the Lumen-Side Domain of Cytochrome *f*

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The following findings concerning the structure of the cytochrome b_6f complex and its component polypeptides, cyt b_6, subunit IV and cytochrome *f* subunit are discussed:

(1) Comparison of the amino acid sequences of 13 and 16 cytochrome b_6 and subunit IV polypeptides, respectively, led to (a) reconsideration of the helix lengths and probable interface regions, (b) identification of two likely surface-seeking helices in cyt b_6 and one in SU IV, and (c) documentation of a high degree of sequence invariance compared to the mitochondrial cytochrome. The extent of identity is particularly high (88% for conserved and pseudo-conserved residues) in the segments of cyt b_6 predicted to be extrinsic on the *n*-side of the membrane.

(2) The intramembrane attractive forces between *trans*-membrane helices that normally stabilize the packing of integral membrane proteins are relatively weak.

(3) The complex isolated in dimeric form has been visualized, along with isolated monomer, by electron microscopy. The isolated dimer is much more active than the monomer, is the major form of the complex isolated and purified from chloroplasts, and is inferred to be a functional form in the membrane.

(4) The isolated cyt b_6f complex contains one molecule of chlorophyll *a*.

(5) The structure of the 252 residue lumen-side domain of cytochrome *f* isolated from turnip chloroplasts has been solved by X-ray diffraction analysis to a resolution of 2.3 Å.

KEY WORDS: Cytochrome bc_1; electron transfer; energy transduction; membrane protein, structure.

INTRODUCTION AND GENERAL CONSIDERATIONS

The cytochrome[2] b_6f complex is one of the three integral oligomeric membrane protein complexes involved in linear or noncyclic electron transport in the chloroplast thylakoid and cyanobacterial membrane systems that participate in oxygenic photosynthesis. It is located electrochemically and in the pathways of electron transport between the two reaction center complexes (Cramer *et al.*, 1991), and its photosynthetic electron transfer reactions can occur in the dark. It is phylogenetically related to the cytochrome bc_1 complex of mitochondria and photosynthetic bacteria (Widger *et al.*, 1984), with which there are many structure–function similarities.

Reviews have recently appeared on sequence–structure–function of the mitochondrial bc_1 complex (Degli Esposti *et al.*, 1993), mutational and mutagenesis studies of the bacterial bc_1 complex (Gennis *et al.*, 1993), and aspects of the function of the cytochrome b_6f complex of oxygenic photosynthesis (Hope, 1993). The present article concerns the cytochrome b_6f complex, new structural information on

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[2] Abbreviations: Cyt, cytochrome; cyt $b(bc_1)$, cytochrome *b* of the bc_1 complex; ESMS, electrospray mass spectrometry; MOA-stilbene, E-β-methoxyacrylate-stilbene; MW, molecular weight; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SU IV, subunit IV; TM, transmembrane.

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the complex, reconsideration of the larger number of compiled sequences of cytochrome b_6 and subunit IV, and a discussion of the crystal structure determination of the cytochrome *f* subunit. It does not consider questions of structure–function related to the Rieske iron-sulfur protein subunit of the complex, aspects of which have been recently considered for the mitochondrial protein (Graham *et al.*, 1993; Link *et al.*, 1993). The present article has the underlying viewpoint that the set of data obtained on cytochrome bc_1 complexes is not sufficient to describe all important properties related to the function of the b_6f complex in oxygenic photosynthesis.

The four major ($M_r > 15,000$) subunit polypeptides of the cytochrome b_6f complex that are readily detected using SDS-PAGE are cytochrome *f* (285 residues in spinach chloroplasts; MW = 31,372), cytochrome b_6 (214 residues; MW = 24,038), the Rieske iron-sulfur protein (180 residues; MW = 18,922), and subunit IV (160 residues; MW = 17,444) (MW data for subunits from different sources compiled in Widger and Cramer, 1991). There are also 4–5 small ($M_r < 5,000$) subunits in the complex, one of which has been identified as a 37-residue *pet*G subunit V (Haley and Bogorad, 1989), whose function is not known.

SEQUENCE COMPARISONS AND STRUCTURAL INFERENCES FOR CYT b_6 AND SUBUNIT IV

The assumption of major subunit and prosthetic and redox group similarity of the cytochrome b_6f complex of oxygenic photosynthetic membranes to the cytochrome bc_1 complex of mitochondria and purple photosynthetic bacteria (Hauska *et al.*, 1983) was extended by the demonstration of significant amino acid sequence identity and hydrophobic segment alignment of the cyt *b* polypeptides (Widger *et al.*, 1984). Hydropathy graphs derived from the amino sequence of the long hydrophobic putative membrane-spanning segments in the N-terminal half of the approximately 400-residue cyt $b(bc_1)$ could be aligned by cross-correlation analysis (Shiver *et al.*, 1989) with a similar graph derived from the 214-residue spinach chloroplast cyt b_6 sequence to (i) identify the four histidine residues apparently conserved and involved in heme coordination, and (ii) infer that cytochrome b_6 is analogous to the heme-binding domain of cytochrome *b* of the bc_1 complex, and subunit IV to its C-terminal half (Widger *et al.*, 1984). This also implied that the information in the single cyt *b* gene product of the bc_1 complex is contained in the split gene products, cyt b_6 and subunit IV, of oxygenic photosynthesis. These genes are contiguous or close in higher plant chloroplasts and cyanobacteria, but separated by a large distance in the green alga *Chlamydomonas* (Büschlen *et al.*, 1991).

The involvement of the same four histidine residues in ligation of the two hemes was also inferred from their conservation in six mitochondrial sequences (Saraste, 1984). The original models for folding of the cytochrome *b* heme binding domain across the membrane bilayer contained five *trans*-membrane helices, with heme coordination involving the second and fifth helices from the N-terminus (Widger *et al.*, 1984; Saraste, 1984). It was subsequently proposed that the fourth helix in this model was not *trans*-membrane, but rather a surface-bound amphiphilic helix (Crofts *et al.*, 1987). Support for the revised four-helix model of the cytochrome *b* heme-binding domain was provided for cyt $b(bc_1)$ by: (i) the consistent mapping pattern of cytochrome *b* mutants resistant to inhibitors known to act primarily at the quinone binding site on the *n*- (e.g., antimycin, funiculosin) and *p*- (e.g., stigmatellin, mythathiazol) sides (cf. discussion of notation in legend to Fig. 1) of the membrane (diRago and Colson, 1988; Howell and Gilbert, 1988; Daldal *et al.*, 1989; Gennis *et al.*, 1993); (ii) the location of both polypeptide termini of cyt b_6 on the *n*-side of the membrane using protease accessibility of epitopes for peptide-directed antibodies (Szczepaniak and Cramer, 1990).

The original calculations of the approximate location and identity of the hydrophobic *trans*-membrane helices (Widger *et al.*, 1984; Saraste, 1984; Crofts *et al.*, 1987) could not predict the orientation of the cyt *b* polypeptide in the membrane. For cyt $b(bc_1)$, the orientation was subsequently established through (i) the distribution of sites of resistance for the *n*- and *p*-side inhibitors mentioned above, and (ii) for cyt *b* of the complex in the bacterium *Rb. sphaeroides*, the sites of fusion to alkaline phosphatase (Yun *et al.*, 1991). (iii) For cyt b_6, the orientation shown in Fig. 2A was determined by protease accessibility to specific epitopes (Szczepaniak and Cramer, 1990), also mentioned above, and is consistent with the statistical *cis*-positive rule of Von Heijne (1992) [cf. Gavel *et al.* (1991) for a specific application to thylakoid membrane proteins]. This rule states that for intrinsic

membrane proteins the number of positively charged residues located on the side of the membrane (*cis*) from which the protein is imported is larger than the number on the opposite (*trans*) side (i.e., the translocation of positively charged residues across biological membranes is energetically costly).

The number of (Arg + Lys) residues on the *n*-(*cis*) and *p*-(*trans*) sides of cyt b_6 is 8 and 5 (not including the intramembrane Arg-86), and for SU IV it is 8 and 3. This identity of the basic residues participating in this asymmetric distribution is essentially conserved in both cyt b_6 and SU IV. All of the basic residues are conserved in the 13 cyt b_6 sequences shown in Table I with the exception of the substitution of Asn (N) for Lys (K) at position 111 in *Protochorothrix hollandica*. In the case of SU IV, the identity of 8 of the 11 residues contributing to the (+) charge bias is conserved (Table IB), *P. hollandica* has the two changes R-15 → L and K-20 → Q, the cyanobacterium *A. quadruplicatum* is also changed at the latter position, and 4 of 14 sequences are changed at position 367. [Note that the *cis*-positive rule does not apply to cytochrome *f* and the Rieske iron-sulfur protein of the cyt b_6f complex because in each of these subunits the peripheral segment of the protein exceeds 60 residues (Von Heijne, 1992); in the case of the Rieske protein, there is also a question as to whether it has a membrane span or is totally extrinsic (Gonzalez-Halphen *et al.*, 1988; Breyton *et al.*, 1994).]

The four-helix model applied to cytochrome b_6 is shown in Fig. 1A along with the folding model for SU IV. The helices of cyt b_6 (Fig. 1A; in the rectangular boxes) and subunit IV (Fig. 1B) are designated A–D and E–F, respectively, and the *n*- and *p*-side extrinsic linking peptide segments "ab," "bc," and "cd" (segments of cyt b_6), and "ef," "fg" (segments of SU IV) according to the notation of Crofts *et al.* (1990). The models of Fig. 1A, B differ from earlier models of cyt b_6 and SU IV (Widger and Cramer, 1991) by (i) inclusion of the tendency of the *p*-side surface segments "ab," "cd" (cyt b_6), and "ef" (SU IV) to form amphiphilic helices. The hydrophobic moments of helices "ab," "cd," and "ef" are 0.39, 0.53, and 0.45, respectively, using a segment length of 15 residues and the amino acid hydrophobicity data base of Eisenberg (1984), as in Shiver *et al.* (1989). This amphiphilic character has been noted previously for the "cd" helix of cyt $b(bc_1)$ (Crofts *et al.*, 1987), and the latter amphiphilic helix has been explicitly included in models of the *p*-side quinone binding site (Robertson *et al.*, 1990). Asp-155 or Glu-166 in the "cd" segment have been proposed to bind DCCD and to facilitate H^+ translocation to the membrane surface associated with *p*-side quinol oxidation (Beattie, 1993). The "ef" segment in SU IV contains seven proline residues, three of which have been included in an "ef" helix because of its pronounced amphiphilicity. Precedents for such a helix are provided by surface helices in the photosynthetic bacterial reaction center: the "ab" helix with three or two (*Rb. capsulatus*), and the "cd" helix with three or two (*C. aurantiacus*) proline residues. (ii) The length of the F, G helices of SU IV has been extended one turn in order to take into account the tendency of basic residues Lys-333 and R-339, R-340 to provide punctuation for TM helices. (iii) The tendency of aromatic residues, and tryptophans in particular, to be found near the aqueous interface (Deisenhofer and Michel, 1989; Jacobs and White, 1989) was taken into account by placing Trp-79 on the *p*-side of helix B, and Trp-246 on the *n*-side of helix E in contact with the bilayer phase.

The orientation of the "fg" helix of subunit IV inferred by Li *et al.* (1991) from trypsin accessibility studies was opposite to that shown in Fig. 1B. The distribution of (Arg + Lys) in a SU IV with an orientation reversed from that shown in Fig. 1B would be decidedly opposite to that predicted by the *cis*-positive rule.

Two striking features of the comparison of sequences of the 13 cyt b_6 and 16 SU IV polypeptides are (a) the high degree of overall sequence identity, as noted in a comparison of an initial set of three complete, and two partial b_6-SU IV sequences (Hauska *et al.*, 1988), and (b) the higher extent of identity on the *n*-side compared to the *p*-side of the membrane. The degree of conservation of the 13 sequences of the 214 cyt b_6 polypeptide (Table IA) is 69% and 79% for identical and pseudo-identical (K ↔ R, D ↔ E, and S ↔ T) residues, and is 53% and 63%, respectively, for the 160 residue subunit IV (Table IB). These levels of identity appear to far exceed those seen in cyt *b* of the mitochondrial bc_1 complex (Hauska *et al.*, 1988; Degli Esposti *et al.*, 1993). In the 18 mitochondrial sequences compiled by Hauska *et al.* (1988), 10% of the residues are invariant (Degli Esposti *et al.*, 1993). Degli Esposti *et al.* (1993) have also noted that the result of sequence analysis of the cytochrome *b* protein from more than 800 species, the most extensive analysis for

Table I. Compilation of Aligned Amino Acid Sequences of (A) Cytochrome b_6 and (B) Subunit IV[a]

(A) Amino Acid Sequence Alignment of Cytochrome *b*6

```
                    1                                                  50
AGMEN               MFTKEVTDS.KLYKWFNER LEIQAISDDI SSKYVPPHVN IFYCLGGITL TCFIIQFATG
HOLLANDICA          MFTKQVQES.GVYKWFNDR LEIEAISDDI SSKYVPPHVN IFYCLGGITL VCFIIQFATG
NOSTOC              .......MA.NVYDWFEER LEIQAIAEDV TSKYVPPHVN IFYCLGGITL TCFLIQFATG
SPINACH             .......MS..VYDWFEER LEIQAIADDI TSKYVPPHVN IFYCLGGITL TCFLVQVATG
BARLEY              ........  KVYDWFEER LEIQAIADDI TSKYVPPHVN IFYCLGGITL TCFLVQVATG
MAIZE               ........  KVYDWFEER LEIQAIADDI TSKYVPPHVN IFYCLGGITL TCFLVQVATG
RICE                .......MS.KVYDWFEER LEIQAIADDI TSKYVPPHVN IFYCLGGITL TCFLVQVATG
TOBACCO             .......MSVKVYDWFEER LEIPAIADDI TSKYVPPHVN IFYCLGGITL TCFLVPVATG
WHEAT               .......MS.KVYDWFEER LEIQAIADDI TSKYVPPHVN IFYCLGGITL TCFLVQVATG
LVWORT              .......M.V.VYDWFEER LEIQAIADDI TSKYVPPHVN IFYCLGGITL TCFLVQVATG
GRACILIS            .......MS.RVYDWFEER LEIQAIADDV SSKYVPPHVN IFYCLGGITF TCFIIQVATG
REINHARDTII         .......MS.KVYDWFEER LEIQAIADDI TSKYVPPHVN IFYCIGGITF TCFLVQVATG
PROTOTHECOI         .......MS.KIYDWFEER LEIQSIADDI SSKYVPPHVN IFYCFGGITF TCFLVQVATG
                                                                HELIX A

                    51                                                 110
AGMEN               FAMTFYYKPT VAEAFTSVQY IMNEVNFGWL IRSIHRWSAS MMVLMMILHI FRVYLTGGFK
HOLLANDICA          FAMTFYYKPS VTEAFTSVQY LMNEVSFGWL IRSIHRWSAS MMVLMMILHV FRVYLTGGFK
NOSTOC              FAMTFYYKPT VAEAFSSVEY IMNEVNFGWL IRSIHRWSAS MMVLMMILHV FRVYLTGGFK
SPINACH             FAMTFYYRPT VTDAFASVQY IMTEVNFGWL IRSVHRWSAS MMVLMMILHV FRVYLTGGFK
BARLEY              FAMTFYYRPT VTEAFSSVQY IMTEANFGWL IRSVHRWSAS MMVLMMILHV FRVYLTGGFK
MAIZE               FAMTFYYRPT VTEAFSSVQY IMTEANFGWL IRSVHRWSAS MMVLMMILHV FRVYLTGGFK
RICE                FAMTFYYRPT VTEAFSSVQY IMTEANFGWL IRSVHRWSAS MMVLMMILHV FRVYLTGGFK
TOBACCO             FAMTFYYRPT VTEAFASVPY IMTEANFGWL IRSVHRWSAS MMVLMMILHV FRVYLTGGFK
WHEAT               FAMTFYYRPT VTEAFSSVQY IMTEANFGWL IRSVHRWSAS MMVLMMILHV FRVYLTGGFK
LVWORT              FAMTFYYRPT VTEAFSSVQY IMTEVNFGWL IRSVHRWSAS MMVLMMILHI FRVYLTGGFK
GRACILIS            FAMTFYYRPT VTEAFLSVKY IMNEVNFGWL IRSIHRWSAS MMVLMMILHV CRVYLTGGFK
REINHARDTII         FAMTFYYRPT VAEAFASVQY IMTDVNFGWL IRSIHRWSAS MMVLMMVLHV FRVYLTGGFK
PROTOTHECOI         FAMTFYYRPT VAEAFTSVQY LMTQVNFGWL IRSIHRWSAS MMVLMMILHI FRVYLTGGFK
                    ----------                          HELIX B

                   111                                                 170
AGMEN               RPRELTWITG VIMATITVSF GVTGYSLPWD QVGYWAVKIV SGVPAAIPVV GDQMVELLRG
HOLLANDICA          NPRELTWITG VILAVITVSF GVTGYSLPWD QVGYWAVKIV SGVPEAIPLV GPLMVELIRG
NOSTOC              KPRELTWVSG VILAVITVSF GVTGYSLPWD QVGYWAVKIV SGVPEAIPVV GVLISDLLRG
SPINACH             KPRELTWVTG VVLGVLTASF GVTGYSLPWD QIGYWAVKIV TGVPDAIPVI GSPLVELLRG
BARLEY              KPRELTWVTG VVLAVLTASF GVTGYSLPWD QIGYWAVKIV TGVPDAIPVI GSPLVELLRG
MAIZE               KPRELTWVTG VVLAVLTASF GVTGYSLPWD QIGYWAVKIV TGVPEAIPVI GSPLVELLRG
RICE                KPRELTWVTG VVLAVLTASF GVTGYSLPWD QIGYWAVKIV TGVPDAIPVI GSPLVELLRG
TOBACCO             KPRELTWVTG VVLAVLTASF GVTGYSLPWD PVGYWAVKIV TGVPDAIPVI GSPLVELLRG
WHEAT               KPRELTWVTG VVLAVLTASF GVTGYSLPWD QIGYWAVKIV TGVPDAIPVI GSPLVELLRG
LVWORT              KPRELTWVTG VILAVLTVSF GVTGYSLPWD QIGYWAVKIV TGVPEAIPII GSPLVELLRG
GRACILIS            KPRELTWVTG IILAILTVSF GVTGYSLPWD QVGYWAVKIV TGVPEAIPLI GNFIVELLRG
REINHARDTII         RPRELTWVTG VIMAVCTVSF GVTGYSLPWD QVGYWAVKIV TGVPDAIPGV GGFIVELLRG
PROTOTHECOI         KPRELTWVTG VLMAVCTVSF GVTGYSLPWD QIGYWAVKIV TGVPDAIPVI GQVLLELLRG
                    ----------                          HELIX C
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Table I. Continued.

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            171                                                    214
AGMEN       GASVGQATLT RFYSLHTFVL PWLIAVFMLA HFLMIRKQGI SGPL*
HOLLANDICA  SASVGQATLT RFYSLHTFVL PWFIAVFMLM HFLMIRKQGI SGPL*
NOSTOC      GSSVGQATLT RYYSAHTFVL PWLIAVFMLF HFLMIRKQGI SGPL*
SPINACH     SASVGQSTLT RFYSLHTFVL PLLTAVFMLM HFLMIRKQGI SGPL*
BARLEY      SASVGQSTLT RFYSLHTFVL PLLTAVFMLM HFPMIRKQGI SGPL*
MAIZE       SASVGQSTLT RFYSLHTFVL PLLTAVFMLM HFPMIRKQGI SGPL*
RICE        SASVGQSTLT RFYSLHTFVL PLLTAVFMLM HFLMIRKQGI SGPL*
WHEAT       SASVGQSTLT RFYSLHTFVL PLLTAVFMLM HFPMIRKQGI SGPL*
TOBACCO     SASVGPSTLT RFYSLHTFVL PLLTAVFMLM HFLMIRKPGI SGPL*
LVWORT      SVSVGQSTLT RFYSLHTFVL PLLTAIFMLM HFLMIRKQGI SGPL*
GRACILIS    SVSVGQSTLT RFYSLHTFVL PLLTATFMLG HFLMIRKQGI SGPL*
REINHARDTII GVGVGQATLT RFYSLHTFVL PLLTAVFMLM HFLMIRKQGI SGPL*
PROTOTHECOI GVAVGQSTLT RFYSLHTFVL PLFTAVFMLM HFLMIRKQGI SGPL*
                       ----------------------------------
                                  HELIX D
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(B) Amino Acid Sequence Alignment of Subunit IV

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            215                                                    264
SPINACH     MGVTKKPDLN DPVLRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
BARLEY      MGVTKKPDLN DPVLRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
MAIZE       MGVTKKPDLN DPVLRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
RICE        MGVTKKPDLN DPVLRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
TOBACCO     MGVTKKPDLN DPVLRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
PEA         MG--------- ---------- ---HNYYGEP AWPNDLLYIF PVVILGTIAC
WHEAT       MGVTKKPDLN DPVLRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
LVWORT      MGVTKKPDLS DPILRAKLAK GMGHNYYGEP AWPNDLLYIF PVVILGTIAC
REINHARDTII MSVTKKPDLS DPVLKAKLAK GMGHNTYGEP AWPNDLLYMF PVVILGTFAC
EUGAMETOS   MSVTKKPDLN DPVLRAKLAK GFGHNTYGEP AWPNDLLYIF PVVIFGTFAC
PROTOTHECOI MAVTKKPDLS DPQLRAKLAK GMGHNYYGEP AWPNDIFYMF PVVIFGTFAG
AGMEN       MSIMKKPDLS DPKLRAKLAQ NMGHNYYGEP AWPNDILFTF PICIAGTIGL
S. 6803     MSIIKKPDLS DPDLRAKLAK GMGHNYYGEP AWPNDILYMF PICILGALGL
NOSTOC      MATQKKPDLS DPQLRAKLAK GMGHNYYGEP AWPNDLLYVF PIVIMGSFAA
HOLLANDICA  MSVLKKPDLT DPVLLEKLAQ NMGHNYYGEP AWPNDLLYTF PVVILGTLAC
S. obliquus MSVTKKPDLT DPVLKEKFAK GMGHNYYGEP AWPNDLLYIF PVVILGTFAC
                                                       ----------
                                                        HELIX E

            265                                                    314
SPINACH     NVGLAVLEPS MIGEPADPFA TPLEILPEWY FFPVFQILRT VPNKLLGVLL
BARLEY      NVGLAVLEPS MIGEPADPFA TPLEILPEWY FFPVFQILRT V---------
MAIZE       NVGLAVLEPS MIGEPAD--- ---------- ---------- ----------
RICE        NVGLAVLEPS MIGEPADPFA TPLEILPEWY FFPVFQILRT VPNKLLGVLL
TOBACCO     NVGLAVLEPS MIGEPADPFA TPLEILPEWY FFPVFQILRT VPNKLLGVLL
PEA         NVGLAVLEPS MIGEPADPFA TPLEILPEWY FFPVFQILRT VPNKLLGVLL
WHEAT       NVGLAVLEPS MIGEPADPFA TPLEILPEWY FFPVFQILRT VPNKLLGVLL
LVWORT      TVGLAVLEPS MIGEPANPFA TPLEILPEWY FFPVFQILRT VPNKLLGVLL
REINHARDTII VIGLSVLDPA AMGEPANPFA TPLEILPEWY FYPVFQILRV VPNKLLGVLL
EUGAMETOS   CIGLAVLDPA AMGEPANPFA TPLEILPEWY FYPVFQILRT VPNKLLGVLA
PROTOTHECOI VIGLAVLDPA AIGEPANPFA TPLEILPEWY FYPVFQLLRT VPNKLLGVLL
AGMEN       ITGLAILDPA MIGEPGNPFA TPLEILPEWY LYPVFQILRV LPNKLLGIAC
S.6803      IAGLAILDPA MIGEPADPFA TPLEILPEWY LYPTFQILRI LPNKLLGIAG
NOSTOC      IVALAVLDPA MTGEPANPFA TPLEILPEWY LYPVFQILRS LPNKLLGVLA
HOLLANDICA  VVGLAVLDPA MVGEPANPFA TPLEILPEWY LYPAFQILRV VPNKLLGILL
S. obliquus VIGLSVLDPA AIGEPANPFA TPLEILPEWY FYPVFQLLRT VPNKLLGVLL
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Table I. Continued.

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                315                                                    364
SPINACH         MASVPAGLLT VPFLENVNKF QNPFRRPVAT TVFLVGTVVA LWLGIGATLP
BARLEY          ---------- ---------- ---------- ---------- ----------
MAIZE           ---------- ---------- ---------- ---------- ----------
RICE            MVSVPTGLLT VPFLENVNKF QNPFRRPVAT TVFLIGTAVA LWLGIGATLP
TOBACCO         MVSVPAGLLT VPFLENVNKF QNPFRRPVAT TVFLIGTAVA LWLGIGATLP
PEA             MVSVPAGLLT VPFLENVNKF QNPFRRPVAT TVFLIGTVVA LWLGIGATLP
WHEAT           MVSVPTGLFT VPFLENVNKF QNPFRRPVAT TVFLIGTVVA LWLGIGATLP
LVWORT          MAAVPAGLLT VPFLENVNKF QNPFRRPVAT TVFLIGTVVA LWLGIGAALP
REINHARDTII     MAAVPAGLIT VPFIESINKF QNPYRRPIAT ILFLLGTLVA VWLGIGSTFP
EUGAMETOS       MAAVPVGLLT VPFIESINKF QNPYRRPIAT ILFLVGTLVA VWLGIGATFP
PROTOTHECOI     MAAVPAGLIT VPFIKIYNKF QNPFRRPVAT TVFLVGTVAA IWLGIGAALP
AGMEN           QGAIPLGLMM VPFIESVNKF QNPFRRPVAM AVFLFGTAVT LWLGAGACFP
S.6803          MAAIPLGLML VPFIESVNKF QNPFRRPIAM TVFLFGTAAA LWLGAGATFP
NOSTOC          MASVPLGLIL VPFIENVNKF QNPFRRPVAT TVFLFGTLVT LWLGIGAALP
HOLLANDICA      QTAIPLGLML VPFIENINKF QNPFRRPIAM AVFLFGTLVT LWMGVAATLP
S. obliquus     MAAVPAGLIT VPFIENINKF QNPYRRPIAT TLFLVGTLVA VWLGIGATLP
                   HELIX F                              HELIX G

                365       374
SPINACH         IDKSLTLGLF*
BARLEY          ----------*
MAIZE           ----------*
RICE            IEKSLTLGLF*
TOBACCO         IDKSLTLGLF*
PEA             IEKSLTLGLF*
WHEAT           IDKSLTLGLF*
LVWORT          IDKSLTLGLF*
REINHARDTII     IDISLTLGLF*
EUGAMETOS       IDISLTLGLF*
PROTOTHECOI     IDISLTLGLF*
AGMEN           IDESLTLGLF*
S.6803          IDKSLTLGLF*
NOSTOC          LDKSLTLGLF*
HOLLANDICA      IDKFFTLGLF*
OOCSP           IEISLTFGLF*
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[a] AGMEN (*Agmenellum quadruplicatum*. Brand *et al.*, 1992); NOSTOC (Cyanobacterium *Nostoc* strain PCC 7906. Kallas *et al.*, 1988); HOLLANDICA (*Prochlorothrix hollandica*. Greer and Golden, 1992); BARLEY (*Hordeum vulgare*. Reverdatto *et al.*, 1989); SPINACH (*Spinacia oleracea*, Heinemeyer *et al.*, 1984); WHEAT (*Triticum aestivum*. Hird *et al.*, 1991); MAIZE (*Zea mays*. Rock *et al.*, 1987, PROTOTHECOI (*Chlorella protothecoides*. Reimann and Kueck, 1989); RICE (*Oryza sativa*. Cote *et al.*, 1988; Hiratsuka *et al.*, 1989); TOBACCO (*Nicotiana tabacum*. Shinozaki *et al.*, 1986); GRACILIS (*Euglena gracilis*. Schlunegger and Stutz, 1984); S. 6803 (*Synechocystis* sp. PCC 6803. Osiewacz, 1992); EUGAMETOS (*Chlamydomonas eugametos*. Turmel *et al.*, 1989); REINHARDTII (*Chlamydomonas reinhardtii*. Büschlen *et al.*, 1991); LVWORT (*Marchantia polymorpha*. Fukuzawa *et al.*, 1987); PEA (*Pisum sativum*. Phillips and Gray, 1984); S. obliquus (*Scenedesmus obliquus*. Kück, 1989).

any integral membrane protein, is that there are only 9 invariant amino acids (Table II).

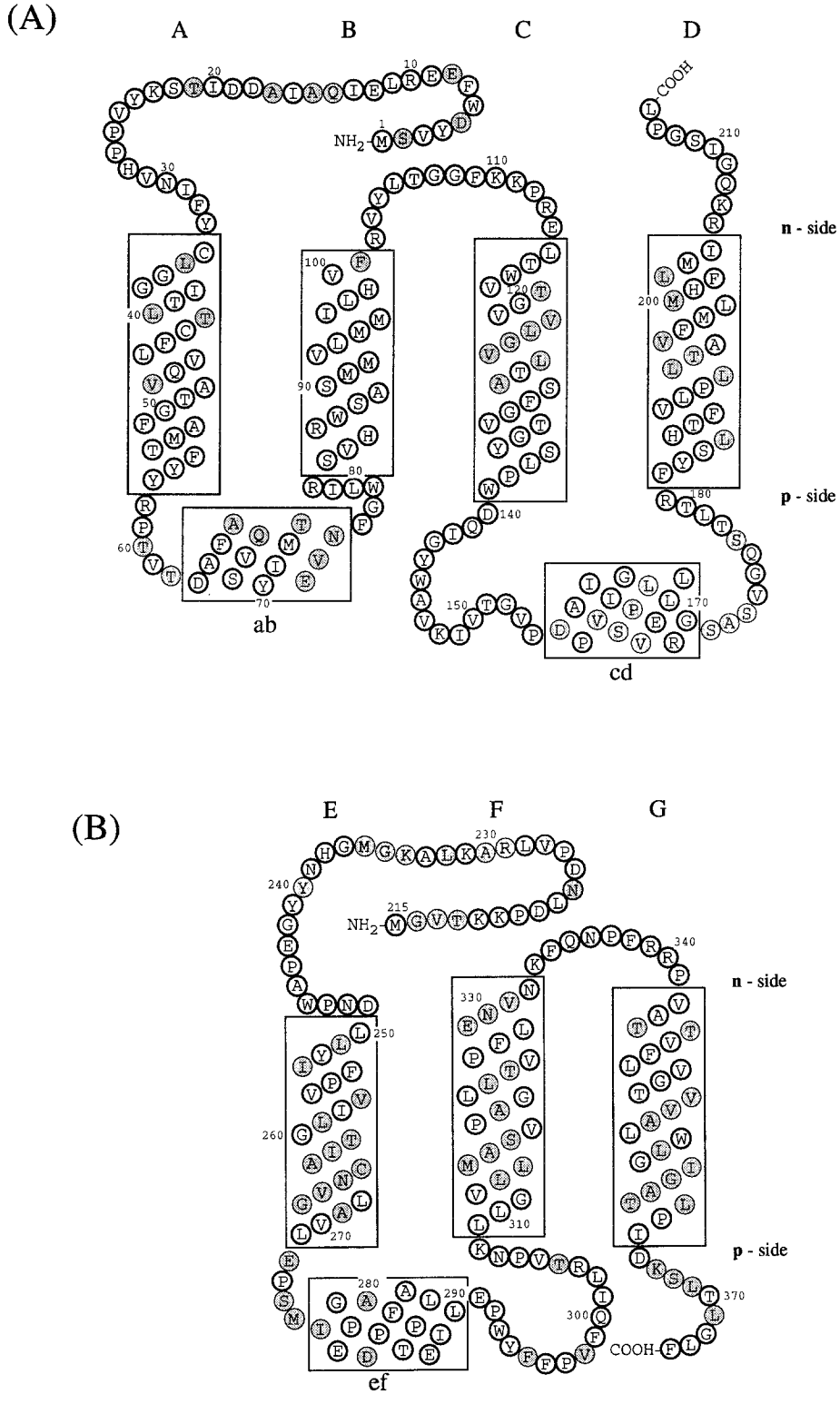
Table II summarizes (a) the position in the sequence of cyt b_6-SU IV, and (b) the likely or proposed function of the 24 most highly conserved residues in the mitochondrial cytochrome *b* (Degli Esposti *et al.*, 1993). Sixteen of the 24 residues are in cyt b_6, according to Degli Esposti *et al.* (1993). Eight are in SU IV, of which three are the consecutive Pro 291-Glu 292-Trp 293. Five of the residues listed as

most highly conserved by Degli Esposti *et al.* (1993) are different although conserved in cyt b_6, where they are Val-132 (*p*-side, C helix), Gly-143 (*p*-side, "cd" loop), Lys-148 (*p*-side, "cd" helix), Ile-149 (*p*-side, "cd" helix), and Arg-206 (*n*-side, C-terminal segment). No such residues occur in SU IV. Thus, four of the 16 most conserved residues in cyt b_6, and four of the five that break the invariance of the superfamily, are in the "cd" segment of cyt b_6. The fifth residue that breaks the invariance, Arg-206, is analogous to His-202 that is otherwise completely conserved in the mitochondrial and photosynthetic bacterial cytochrome. In the latter, it is His-217. The mutation His 217 → Arg in the bacteria results in an inhibition of the rate of heme b_n oxidation by a factor of 3–4 (Gray and Daldal, 1993; Hacker *et al.*, 1993). The fact that the Arg residue that results in inhibition of bacterial heme b_n (cf. discussion of notation in legend of Fig. 1) oxidation is conserved in cyt b_6 can be conservatively interpreted as indicating that the putative quinone binding site on the *n*-side of the membrane (Q_n site) has a different structure compared to that in the mitochondria and bacteria. Arg 206-Lys 207 have also been shown to be accessible to trypsin in thylakoid membranes (Szczepaniak and Cramer, 1990). This implies that the heme coordinated by His-201 is close (ca. 7–9 Å) to the *n*-side interface, in contrast to heme b_n of the mitochondrial cytochrome that was inferred from EPR experiments with external paramagnetic complexes (Ohnishi *et al.*, 1989) and electrochromic msec "slow" phase measurements (Glaser and Crofts, 1984; Robertson and Dutton, 1988) to be appreciably removed (approximately 25 Å) from the *n*-side interface. One other Arg residue that is uniquely conserved in the cyt b_6 sequences is Arg-86 adjacent to His-85 in the B helix. It is the only conserved residue that carries a nominal charge in the designated *trans*-membrane helices of the cytochrome *b* polypeptides.

The difference in sequence and probable structure of the *n*-side of cyt b_6 compared to cyt $b(bc_1)$ is further illustrated by the extremely high level of sequence identity of the *n*-side peripheral segments of cyt b_6, 78% and 87% for invariance and pseudo-invariance (Table IA, Fig. 1). These levels are higher than the corresponding values of 61% and 73% for the *p*-side domains. Thirteen of the last fifteen residues in cyt b_6, Met-200 to Leu-214 on the *n*-side of helix D, are invariant, with Arg-206 approximately in the middle of this segment. The greater invariance on the *n*-side of the membrane is a bias opposite to that in cytochrome *b* of the bc_1 complex (Degli Esposti *et al.*, 1993). The *n*-side of the b_6f complex is known to be characterized by the lack of a high-affinity binding site for quinone-analogue inhibitors (Widger and Cramer, 1991; Hope, 1993). The lack of such a site for a potent *n*-side inhibitor such as antimycin A is one of the obvious differences between the b_6f and bc_1 complexes, and has been a major point in discussions about the possibility of different properties of the b_6f complex compared to cyt $b(bc_1)$ (Cramer *et al.*, 1991; Rich *et al.*, 1992). [The inhibitors NQNO (Selak and Whitmarsh, 1982; Rich *et al.*, 1991; Kramer and Crofts, 1992) and MOA-stilbene (Rich *et al.*, 1992) may be weakly binding *n*-side inhibitors of cyt b_6; see Hope (1993) for a discussion of some inconsistencies in this interpretation regarding NQNO.] However, when contrasted with mitochondria, the higher extent of sequence identity of the *n*-side of cyt b_6 and SU IV implies that (i) a much higher degree of sequence invariance is required to establish a binding site of plastoquinone to allow the same Q-cycle mechanism that has been documented for mitochondria and bacterial chromatophores (Crofts, 1985; Trumpower, 1990); (ii) alternatively, the *n*-side extrinsic regions of cyt b_6 and SU IV are responsible for essential functions in addition to, or instead of, the Q cycle function. One such function might be the docking of redox proteins [e.g., the "G" protein component of Joliot and Joliot (1988)] that can supply reducing equivalents to heme b_n from the *n*-side aqueous phase.

WEAKNESS OF HELIX–HELIX INTERACTIONS

The b_6f complex is extracted from the membrane as an integral membrane protein complex in detergents such as cholate, MEGA-9, or β-D-dodecylmaltoside. As a membrane protein complex, the b_6f complex has the unusual property that the subunits can be separated from the complex and from each other at alkaline pH (ca. 10.5–11.5) [Fig. 2; Szczepaniak *et al.*, 1991; Cramer *et al.*, 1992]. This implies that, unlike membrane proteins such as bacteriorhodopsin (Popot and Engelman, 1990) and the glycophorin dimer (Lemmon *et al.*, 1992), the attractive forces arising from intramembrane hydrophobic, hydrogen bond, and van der Waals interactions between the subunits are relatively weak. As a consequence, the electrostatic repulsive force


(A)
A
B
C
D
NH2-
COOH
n - side
p - side
ab
cd
(B)
E
F
G
NH2-
COOH-
n - side
p - side
ef

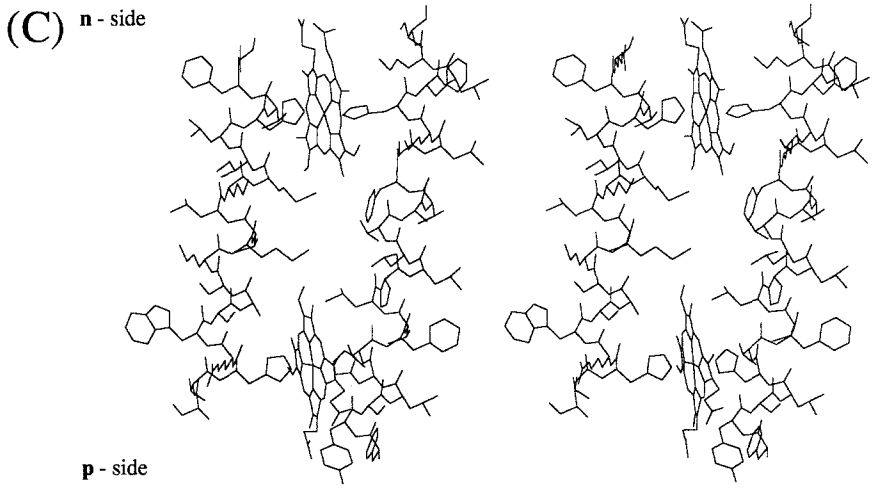


Fig. 1. Folding pattern across the thylakoid membrane of (A) cytochrome b_6 and (B) subunit IV predicted by the distribution and conservation in the 13 and 16 sequences shown in Table IA, B of long (ca. 20 residue) hydrophobic segments. The hydrophobic membrane bilayer is delineated by the rectangular boxes. Hatched circles represent residues that are not invariant or pseudo-invariant. (C) Molecular model (stereo view) of heme-bridged helices B and D of spinach cyt b_6. The amino acid sequences of these helices are: B helix, NH_2 (*p*-side)- SVHRWSASMMVLM-MILHVF-COOH; D helix, NH_2 (*p*-side)-FYSLHTFVLPLLTAVFMLMHFLMI-COOH. *Nomenclature*: (1) The two sides of the membrane are designated *n*- and *p*-, the electrochemically negative and positive sides, which correspond to stroma and lumen, respectively, in the case of chloroplast thylakoid membranes. This notation is chosen instead of *i* and *o* proposed in the recent review of Degli Esposti *et al.* (1993) because it is conceptually simple, which is important for teaching purposes, allows comparative discussion of bacteria, chloroplasts, and mitochondria without reference to the definition of all of the respective extramembrane compartments (lumen, matrix, cytoplasmic, stroma, intermembrane, periplasmic), and is unambiguous, unlike *i* and *o* which can readily be mistaken by nonspecialists for "inside" and "outside." (2) Putative *trans*-membrane helices and connecting peripheral domains are labeled A–G and ab...fg, respectively, according to the proposal of Crofts *et al.* (1990). (3) Cytochrome *b* hemes on the *n*- and *p*-sides of membrane coordinated by His-99 and His-201 and His-85 and His-186, respectively, bridging helices B and D, are designated hemes b_n and b_p, respectively. The notation is preferred over that of b_h and b_l, for high- and low-potential hemes, proposed by Degli Esposti *et al.* (1993) because (a) there is experimental disagreement for cyt b_6 *in situ* concerning the existence of experimentally resolvable high- and low-potential *b* hemes, and (b) the redox titration of cyt *b* of the bc_1 complex from the PS3 thermophilic bacterium, which shows some similarity to the b_6f complex, does not show a discernible difference in E_m of the two hemes (Kutoh and Sone, 1988). Regarding (a), two reports (Furbacher *et al.*, 1989; Rich *et al.*, 1991) show that the E_m values of the two hemes are not separately resolved ($\Delta E_m < 50\,mV$) in thylakoid membranes in the presence of Mg^{2+}; one (Kramer and Crofts, 1990) showed potentiometric resolution ($b_h = -15\,mV$, $b_l = -110\,mV$, $\Delta E_m = 95\,mV$) of the two hemes. The latter result was obtained in the absence of Mg^{2+} but is in close agreement with the heme potentials measured in the isolated b_6f complex (Hurt and Hauska, 1983; Nitschke *et al.*, 1988). It was proposed (Kramer and Crofts, 1990) that the use of a continuous high-measuring light intensity in the redox titrations of Girvin and Cramer (1984) and Furbacher *et al.* (1989) might account for the discrepancy, but the intensity ($0.6\,\mu E \cdot m^{-2}\,sec^{-1}$) found by Kramer and Crofts (1990) to cause cytochrome oxidation was at least 30 times that used ($10-20\,nE \cdot m^{-2} \cdot sec^{-1}$) by the latter authors.

exerted between the subunits at alkaline pH, arising from net excess negative charge on the peripheral polypeptide segments, is sufficient to cause the subunits of the complex to separate in the membrane. The fact that the subunits of the spinach chloroplast b_6f complex do undergo lateral separation in the membrane at high pH is inferred from the separate and sequential release of the subunits of the complex as a function of increasing pH. A satisfying explanation does not exist as to why the subunits are not just separated, but also extruded from the membrane. One may speculate that the release from the mem-

Table II. Summary of Residues of Cyt b_6 and SU IV in Sequence Compilations of Tables IA, B and Figs. 2A, B That Are Most Highly Conserved (Degli Esposti *et al.*, 1993)

Residue	Invariance	Location (Figs. 2A, B)	Function
		Cytochrome b_6	
Gly 36	Complete[b]	*n*-side, A helix	Heme pocket[a]
Gly 50	Essentially complete[b]	A helix	—
Gly 78	Essentially complete[b]	"ab" region	Turn between "ab" and B helices
Arg 82	(+) Residue essentially complete[b]	*p*-side interface, B helix	Stop transfer, B helix
His 85	Complete[b]	*p*-side, B helix	Ligand of *p*-side heme[c,d]
Ser 90	Incomplete[b]	B helix	—
His 99	Complete[b]	*n*-side, B helix	Ligand of *n*-side heme[c,d]
Arg 102	(+) Residue complete[b]	*n*-side interface, B helix	Stop transfer, B helix
Trp 117	Complete[b]	*n*-side, C helix	—
Gly 120	Essentially complete[b]	*n*-side, C helix	—
Gly 134	Essentially complete[b]	*p*-side, C helix	—
Tyr 135	Incomplete[b]	*p*-side, C helix	—
Trp 145	Incomplete[b]	*p*-side, "cd" helix	—
Thr 178	Essentially complete[b]	*p*-side interface—"cd" helix	—
His 186	Complete[b]	*p*-side, D helix	Ligand of *p*-side heme[c,d]
His 201	Complete[b]	*n*-side, D helix	Ligand of *n*-side heme[c,d] One extra residue between two His ligands on D helix[c]
		SU IV[e]	
Asp 249	Essentially complete[b]	*n*-side interface, E helix	—
Ile 289	Incomplete[b]	*p*-side, "ef" loop	—
Pro 291	Complete[b]	*p*-side, "ef" loop	—
Glu 292	Essentially complete[b]	*p*-side, "ef" loop	—
Trp 293	Essentially complete[b]	*p*-side, "ef" loop	—
Leu 302	Essentially complete[b]	*p*-side, "ef" loop	—
Lys 308	Incomplete[b]	*p*-side interface, F helix	Stop transfer, F helix
Gly 311	Essentially complete[b]	*p*-side interface, F helix	Turn between "ef" and F helix

[a] Tron *et al.*, 1991; Yun *et al.*, 1991; [b] Degli Esposti *et al.*, 1993; [c] Widger *et al.*, 1984; [d] Saraste, 1984; [e] note that the numbering of SU IV is different from that used by Degli Esposti *et al.* (1993) who omitted the first 30 residues of the SU IV sequence. Use of the numbering system of the latter authors would cause overlap of numbering with the cyt b_6 sequence from residue 195–214. The basis of the numbering system used in the present work is based on the concept that SU IV is a functional extension of cyt b_6.

brane is a consequence of the partial deprotonation and neutralization of the basic residues that are proposed to form the defined "stops" for the predicted *trans*-membrane α-helices A, B, and D of cyt b_6 (Fig. 1A) and E, F, and G of SU IV (Fig. 1B). Once the "stops" are lost, the positions of the helices in the membrane would have fewer thermodynamic constraints, and more freedom to move in the direction normal to the plane of the membrane.

The relative weakness of the attractive intramembrane forces between the subunits of the b_6f complex may be partly a consequence of prosthetic groups such as the helix-bridging hemes (Fig. 1C) providing spacers between these helices that prevent close packing between some of the *trans*-membrane helices.

THE b_6f COMPLEX AS A STRUCTURAL AND FUNCTIONAL DIMER

The relatively weak intramembrane forces between subunits could also facilitate interconversion of monomer and dimer forms of the complex. The existence of a dimer form of isolated cyt b_6f reconstituted *in vitro* was indicated from its geometric cross-section in electron micrographs (Mörschel and Staehelin, 1983). The presence of both monomer and dimer forms in isolated cyt b_6f complex was inferred from (a) the presence in a sucrose density gradient of two fractions of unknown, but different, relative molecular weight, (b) the conversion of the higher to the lower M_r form after longer centrifugation times or in higher Triton detergent concentrations, (c) inhibition of this conversion by cross-linking

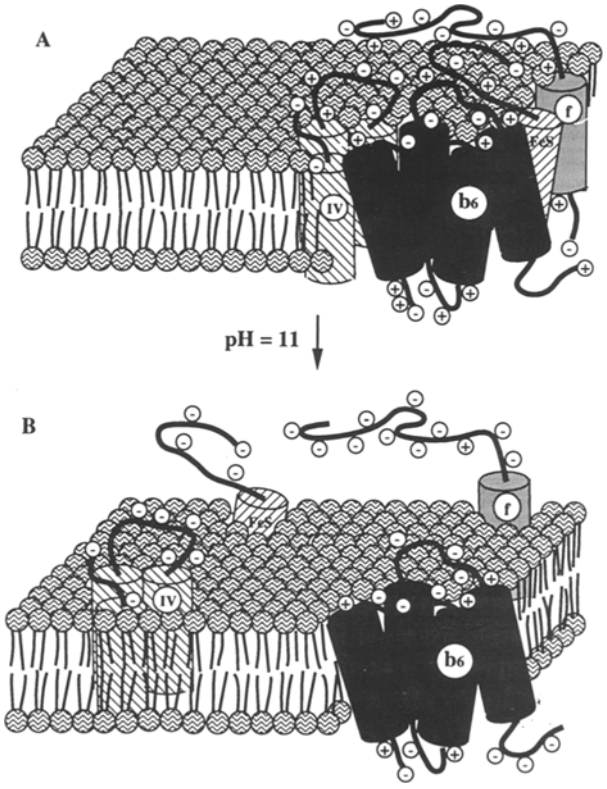


Fig. 2. Schematic diagram of the major subunits of the cytochrome b_6f complex (A) as an organized cluster at neutral ambient pH, and (B) after lateral separation of the subunits in the membrane at alkaline pH (Cramer *et al.*, 1992; figure reproduced with permission of The American Society for Biological Chemistry and Molecular Biology).

reagents, and (d) the generation of different cross-linked M_r forms from the high- and low-M_r fractions (Chain and Malkin, 1991). M_r values and distinct sizes and geometries corresponding to monomer and dimer forms of cyt b_6f complex isolated from spinach thylakoids were determined by (a) FPLC molecular chromatography in β-D-dodecylmaltoside, (b) native gel electrophoresis according to the method of Schägger and von Jagow (1991), and (c) averaged images obtained by electron microscopy of negatively stained single particles (Huang *et al.*, 1993). The dimer was shown to be much more abundant (80–90% of the total complex) than the monomer when assayed by native gel electrophoresis (Huang *et al.*, 1993). When isolated by FPLC molecular sieve chromatography which, compared to the native gel technique, resulted in a higher fraction ($\leq$ 30% of the total) of monomer, the dimer was also much more ($\sim$ 5-fold) active in electron transfer from

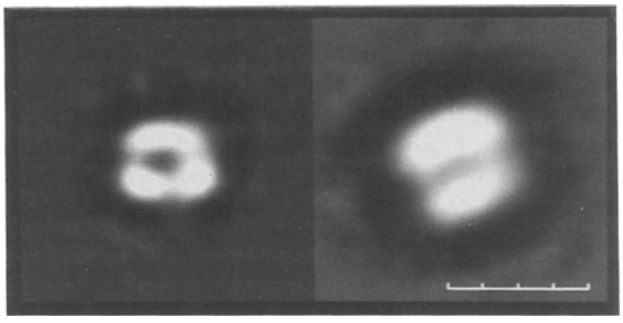


Fig. 3. Electron micrographs of negatively stained (left) monomer and (right) dimer forms of the cytochrome b_6f complex. Monomer and dimer images were computed as the average of 58 and 59 single-particle images, respectively (Huang *et al.*, 1993). Size bar = 10 nm.

plastoquinol-2 to plastocyanin. The level of activity of the monomer was low enough that it could be attributed to residual dimer (Huang *et al.*, 1993). Single-particle images of the monomer and dimer are shown in Fig. 3. A discussion of structure determination by electron microscopy is presented elsewhere in this volume by Boekema *et al.*

The existence of the dimeric form as the predominant form of the purified mitochondrial cytochrome bc_1 complex is well established (Weiss and Kolb, 1979; Von Jagow and Sebald, 1980; Leonard *et al.*, 1981; Nalecz and Azzi, 1985), although the functional significance of this dimer is still unclear (de Vries *et al.*, 1983; Schmitt and Trumpower, 1990; Bechmann *et al.*, 1992; Nieboer and Berden, 1992). It is known that oligomeric structures of membrane proteins are statistically likely in the two-dimensional space of the membrane (Grasberger *et al.*, 1986), and that a dimeric structure and dimer–monomer transitions of the respective receptors are important in the mechanism of *trans*-membrane signaling in at least several systems (Pakula and Simon, 1992; Bormann and Engelman, 1992). Two of the more thoroughly studied examples include the bacterial aspartate chemoreceptor (Milburn *et al.*, 1991), and the epidermal growth factor receptor-tyrosine kinase (Canals, 1992) signaling systems. We make the admittedly speculative suggestion that the cytochrome b_6f and bc_1 complexes are primitive *trans*-membrane signaling systems. In the case of the b_6f complex, this is suggested by its apparent association with a protein kinase, the LHCP kinase, on the *n*-side of the membrane, and the ability of *p*-side quinone analogue inhibitors to block kinase activity (Gal *et al.*, 1990a, b).

MOLECULE OF CHLOROPHYLL *a* IN b_6f COMPLEX

The presence of chlorophyll in the spectrum of the cytochrome b_6f complex isolated from spinach chloroplasts was noted in a benchmark paper on the purification and properties of the complex (Hurt and Hauska, 1983). The cyt b_6f complex from the cyanobacterium *Synechocystis* PCC 6803, which was isolated in the monomeric form, was found to contain a single chlorophyll *a* molecule that was determined by linear dichroism analysis to be oriented in the complex (Bald *et al.*, 1992). Electron microscopic analysis of this complex was carried out by Bald *et al.* (1992) and is reviewed in the present volume of this journal by Boekema *et al.* Analysis of the isolated b_6f complex from spinach chloroplasts showed a chlorophyll *a*:cytochrome *f* stoichiometry > 1, but a smaller value close to 1 when the dimer was further purified from the complex by FPLC molecular sieve chromatography (Huang *et al.*, 1993). The latter stoichiometry appeared to be the minimum value because it was not decreased further by an additional chromatographic step, implying that the one chlorophyll *a* molecule was not bound adventitiously.

CYTOCHROME *f* STRUCTURE: BIOCHEMICAL AND CELL BIOLOGICAL CONSEQUENCES

The understanding of structure/function of cytochrome *f* was advanced recently by the determination of its X-ray structure at a resolution of 2.3 Å (Martinez *et al.*, 1991, 1992, 1993). The structure that was solved was that of the soluble 252-residue lumen-side domain of the 285-residue (MW = 31,298 in turnip chloroplasts) mature polypeptide that spans the membrane once (Fig. 4C).

The use of the lumen-side polypeptide of the cytochrome arose from attempts to prepare intact cytochrome *f* from spinach chloroplast thylakoid membranes for crystallization. These attempts were abandoned because the cytochrome tended to aggregate. This had been noted previously by Ho and Krogmann (1980) who determined a molecular weight of 285,000 for the aggregate that approximately corresponded to an octamer. Preparations of cytochrome *f* from cruciferous plants, however, were reported to be soluble and monomeric with an M_r value of 27,000 instead of 32,000–33,000 (Gray,

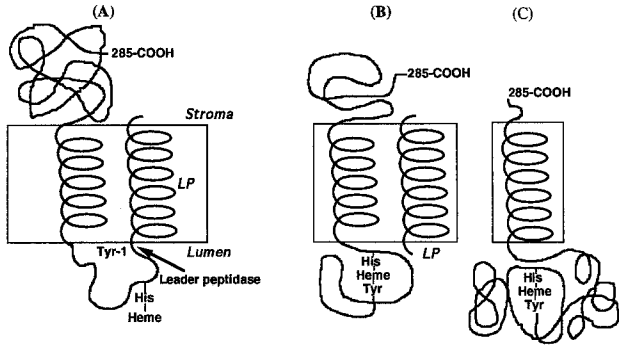


Fig. 4. (A, B) Model for processing and assembly of intermediate forms of cyt *f* and (C) membrane topography of mature form. The schematic shows that complete heme ligation involving the α-amino group of Tyr-1 cannot occur until the 35-residue leader peptide (LP) has been removed by the processing peptidase that is believed to reside on the lumen side of the membrane (Johnson *et al.*, 1991; Kirwin *et al.*, 1991).

1978). It was suggested that the decrease in apparent molecular size might arise from proteolysis at the C-terminus because the N-terminal sequence of cytochrome *f* from the cruciferous plant, rape, was found to be identical to that of spinach and pea (Willey *et al.*, 1984). It was also inferred that the tendency of the spinach cytochrome *f* to aggregate in aqueous solution might arise from the 20-residue hydrophobic segment (residues Nos. 251–270, underlined in Table III) near the C-terminus, and that removal of the hydrophobic domain near the C-terminus might explain the water-soluble monomeric nature of the cruciferous cytochrome *f* fragment. Therefore, it was decided to purify cruciferous cytochrome *f* for structural analysis.

Cytochrome *f* was purified from turnip leaves and found to display an M_r value of 31,000 on SDS-PAGE, compared to 33,800 in the turnip thylakoid membrane (Fig. 5). After promising crystals had been obtained, truncation of the turnip cytochrome *f* near the C-terminus during purification was confirmed by C-terminal sequencing of the turnip cytochrome polypeptide with carboxypeptidases Y and A. Three of the last four residues were found to be Leu 249-Arg 250-Val 251 (Martinez *et al.*, 1991). The fourth C-terminal residue was inferred to be a serine at position 252, instead of the glutamine that was conserved in the several chloroplast cytochrome *f* sequences known at that time. It was therefore thought that the explanation of the susceptibility of cytochrome *f* to lumen-side cleavage by an endogenous protease was a Gln → Ser substitution at residue 252 (Martinez *et al.*, 1991). Determination of

Table III. Amino Acid sequence of Turnip Cytochrome *f* (Gray, 1992).

```
1                                                              50
YPIFAQQNYE NPREATGRIV CANCHLASKP VDIEVPQAVL PDTVFEAVVK

51                                                            100
IPYDMQLKQV LANGKKGALN VGAVLILPEG FELAPPDRIS PEMKEKIGNL

101                                                           150
SFQNYRPNKK NILVIGPVPG QKYSEITFPI LAPDPATNKD VHFLKYPIYV

151                                                           200
GGNRGRGQIY PDGSKSNNTV YNATAGGIIS KILRKEKGGY EITIVDASNE

201                                                           250
RQVIDIIPRG LELLVSEGES IKLDQPLTSN PNVGGFGQGD AEIVLQDPLR

251                                          285
VQGLLFFLGS VVLAQIFLVL KKKQFEKVQL SEMNF*
  ↑
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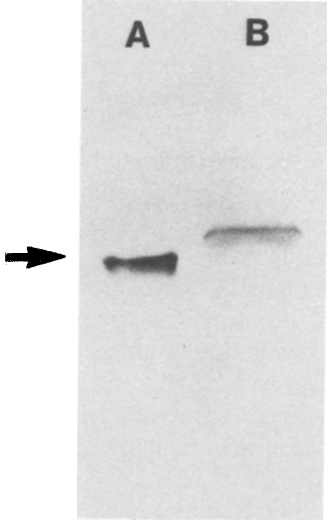

Fig. 5. SDS-PAGE of cytochrome *f* purified from turnip thylakoid membranes (lane A), compared to that of the cytochrome in membranes that were dissolved in SDS and run on the gel without prior extraction (lane B). $\Delta M_r = 3,000$ between the two bands that correspond to polypeptides with (A) the 252 residue turnip cyt *f* fragment (arrow) and (B) the complete turnip cyt *f* polypeptide with 285 residues. The molecular weight of the 252-residue polypeptide is 27,500 including the covalently bound heme. The purification procedure was essentially as described by Gray (1978), except for the use of PMSF (0.5 mM), benzamidine (2 mM), and ϵ-aminocaproic acid (2 mM) (Martinez *et al.*, 1992, 1993). For electrophoresis, the cytochrome was solubilized in 50 mM Tris-HCl, pH 8.6, 4 M urea, 4% SDS, 10% glycerol, and 5% β-mercaptoethanol, and run with a gel system containing 15% acrylamide (Piccioni *et al.*, 1982).

the nucleotide sequence of the turnip cytochrome *f* gene by J. C. Gray showed that residue 252 is, in fact, a glutamine in the turnip (*Brassica campestris*) cytochrome *f* (Gray, 1992). Therefore, the explanation of the "natural" proteolysis in the *Cruciferae* cytochrome *f* is the existence of a protease in chloroplasts from these plants that is activated or made accessible to the lumen-side of the cytochrome *f* during its extraction from the thylakoid membranes. The same cleavage was subsequently reported for cytochrome *f* from charlock (Gray, 1992). It is perhaps interesting that the cleavage predominantly occurs two residues into the 20-residue, hydrophobic *trans*-membrane α-helix. This suggests that the protease might be related to a processing or leader peptidase. The thylakoid processing peptidase of cytochrome *f* is known to be membrane-bound (Johnson *et al.*, 1991), and the active site of that for plastocyanin is on the lumen side of the membrane (Kirwin *et al.*, 1991). On the other hand, the cleavage site of a processing protease should be unique, whereas analysis of the charlock (Gray, 1992) cytochrome *f* polypeptide by ESMS showed that the C-terminus is somewhat ragged, with small populations of fragments resulting from cleavage at residues adjacent to Glu-252. Preliminary ESMS of the turnip cyt *f* fragment indicated that it is $\geq 90\%$ of the fragment population (D. Huang, D.L. Smith, and W.A. Cramer, unpublished data). The residue closest to the C-terminus for which electron density can be resolved in the structure is Leu-249 adjacent to Arg-250 (Fig. 6).

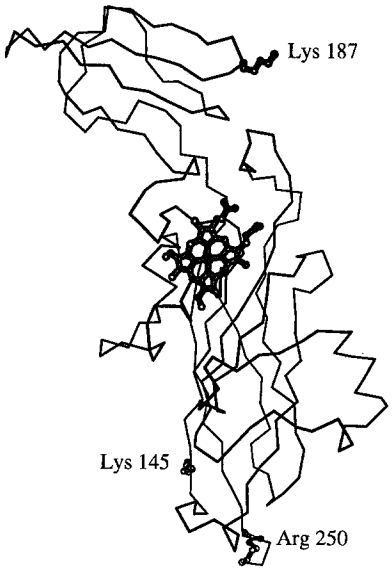

Fig. 6. α-Carbon model of the lumen-side extrinsic polypeptide of turnip (*French*, "le navet"; *German*, "die Steckrübe" or "der Kohlrabi") cytochrome *f* (SDS-PAGE in Fig. 5) showing the large and small domains of the cytochrome, the heme, and residues Lys-145 and Lys-187 in the large and small domains, and Arg-250 at the C-terminus that are separated by 33, 28, and 45 Å, respectively, from the heme iron.

The successful crystallization of the soluble turnip cyt *f* polypeptide that contains a small part (Val 251-Gln 252) of the putative hydrophobic membrane-spanning helical domain suggests a strategy for the systematic genetic engineering for purposes of crystallization of redox proteins such as cytochrome c_1 or the Rieske iron-sulfur protein that have the same kind of general topography: generate a polypeptide fragment of the peripheral soluble fragment that is as long as possible to allow proper folding of the protein. This fragment could include a few residues of the hydrophobic helical domain, in order to increase the chances for correct folding of the fragment without affecting solubility.

The turnip cytochrome *f* polypeptide was crystallized at 5°C in the reduced form in the presence of dithioerythreitol using 40–42% acetone as the precipitant (Martinez *et al.*, 1992). The structure has been solved by multiple isomorphous replacement and anomalous scattering to a resolution of 2.3 Å (Martinez *et al.*, 1993). Some of the major features of the structure are:

1. Unlike the prototypical structure of soluble cytochrome *c* that has one mostly α-helical domain, the elongate ($25 \times 35 \times 75$ Å) cytochrome *f* structure is made of two domains whose major secondary structure motif is a β-sheet.

2. The heme is near the interdomain interface in the larger domain, the Fe 45 Å from the C-terminus of Arg-250 that is connected to the TM α-helix.

3. The smaller domain, consisting of residues 169–231, contains Lys-187, previously shown to cross-link to Asp-44 of plastocyanin (Morand *et al.*, 1989; cf. Redinbo *et al.*, this volume), that is solvent-exposed and 28 Å from the heme Fe (Fig. 6). Lys-187 is in a positively charged region that includes Lys-185 and Arg-209 on the small domain. Lys-66, Lys-65, and Lys-58 are neighboring basic residues on the large domain.

4. The axial sixth heme ligand is the α-amino group of the N-terminal tyrosine residue. A number of spectroscopic studies had noted similarities between spectra of cytochrome *f* and soluble mammalian cytochrome *c* at alkaline pH (> 10) in which the methionine ligand is exchanged for a lysine (Siedow *et al.*, 1980; Davis *et al.*, 1988; Rigby *et al.*, 1988; Simpkin *et al.*, 1989). The conserved Lys-145 was proposed as the axial sixth ligand (Davis *et al.*, 1988). However, the ϵ-amino group of Lys-145 is 33 Å from the heme iron. The amino group function inferred from the spectroscopic studies can be fulfilled by the tyrosine α-amino group, and in one respect is fulfilled even more readily because the pK of the latter is close to neutrality.

5. Unlike mammalian cytochrome *c*, the heme is insulated from the aqueous phase by a shield including three aromatic residues of which one is Tyr-1. This may explain why the midpoint redox potential of cyt *f* is approximately 100 mV more positive than that of mammalian cyt *c*.

Because the structure of plastocyanin is known (Gross, 1993; Redinbo *et al.*, this volume), the question immediately arises as to its competent binding configuration with cytochrome *f*. Information on the sites of interaction was provided by covalent cross-linking of plastocyanin to cytochrome *f* using the water-soluble 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). One site of linkage involved Asp-44 of plastocyanin and Lys-187 of cyt *f*. A second linkage involved Glu-59 and/or 60 to an unknown, presumably basis, residue on cyt *f*. As noted above, Lys-187 is in a basic "patch" of residues (Fig. 6). The transfer of electrons from cytochrome *f* to plastocyanin is thought to be mediated as well by the Tyr-83 residue of plastocyanin which is exposed on the surface of PC and

whose ring is approximately 9–10 Å and 10 Å from Asp-44 and the Cu atom, respectively. The distance of Asp-44 from the copper is 20 Å. With cyt *f* in the orientation shown in Fig. 6, PC would bind through its acidic regions (residues 42–45 and 59–61 of spinach plastocyanin) on the side of the barrel to the basic region of cyt *f* around Lys-187, the copper would be at the bottom of the PC barrel in this binding geometry, and Tyr-83 would then be located closer to the heme than Asp-44. It should be noted that covalent cross-linking of the PC–cyt *f* pair renders it incompetent for intermolecular electron transfer (Qin and Kostic, 1993), and the PC inactive in the reduction of photosystem I (Morand *et al.*, 1989).

Orientation of Cytochrome *f* Relative to the Membrane Surface

It would seem that interaction of cytochrome *f* with the Rieske protein would be facilitated if the long axis of the large domain of cyt *f* was oriented with not too large an angle relative to the plane of the membrane. Interaction between the proposed surface "cd" helix of cytochrome *b* and the analogous cytochrome c_1 is implied by the existence of a second site suppressor mutation in cyt c_1 to a primary inhibitor-resistant mutation in the "cd" loop of cyt $b(bc_1)$ of *Rb. capsulatus* (F. Daldal, personal communication). It has been observed in EPR studies on magnetically oriented chloroplast membranes (Bergstrom and Vanngard, 1982; Crowder *et al.*, 1982) and dehydrated two-dimensional lattices of cytochrome b_6f complex oriented after centrifugation on Mylar sheets (Riedel *et al.*, 1991) that the cyt *f* heme has a broad (Bergstrom and Vanngard, 1982; Riedel *et al.*, 1991) or narrow (Crowder *et al.*, 1982) distribution of orientations relative to the membrane surface. The most probable orientation of the heme plane in both kinds of distributions would have it sharply tilted at an angle of approximately 25–30° relative to the plane of the membrane. Although there may be some flexibility in the orientation of cyt *f*, the most probable orientation of the protein appears to be favorable for interaction with surface-bound or extrinsic segments of the Rieske iron-sulfur protein and cyt b_6.

Sixth Heme Ligand of Cytochrome *f*: Consequences for Protein Translocation and Assembly

The consequence of the sixth heme ligand being the α-amino group of the N-terminal Tyr residue of the mature protein is that the complete ligation of the heme that results in formation of the low-spin coordination state, and assembly of the final folded state of the protein, cannot occur until the signal peptide of the intermediate translocated preprotein has been cleaved by the processing peptidase. This peptidase is believed to be located on the lumen side of the membrane (Johnson *et al.*, 1991; Kirwin *et al.*, 1991). This implies that the protein folding could not be completed until translocation has proceeded at least to the point where a significant part of the cytochrome including Tyr-1 and His-25 has crossed the membrane. In addition, the ligation of the heme and associated folding, as in the translocation pathway of apocytochrome *c* (Pfanner and Neupert, 1990), may provide additional free energy needed to complete the translocation process.

The use of the Tyr-1 α-amino group as the sixth heme ligand can be viewed as a control mechanism to ensure a proper delay in the timing of protein refolding, and is an example of the general principle of membrane protein translocation that the protein to be translocated be unfolded (Verner and Schatz, 1988; Pfanner and Neupert, 1990). Although the use of amino terminal ligation thus seems to be a rational mechanism, it appears that it is not necessarily ubiquitous for metalloproteins even for the closely related cytochrome c_1 protein: (i) in the nitrogen-fixing microaerobic endosymbiont, *Bradyrhizobium japonicum*, cytochromes *b* and c_1 can be made by mutagenesis as a single two-domain polyprotein connected by a 31-residue internal signal peptide (Thony-Meyer *et al.*, 1991). Although it was shown in the latter work that covalent binding of the c_1 heme was required for formation of the bc_1 polyprotein, cleavage of the internal signal peptide was not. The use of a sixth ligand different from the N-terminal α-amino group, for at least some cytochromes c_1, is also suggested by the appearance of a normal reduced α-band spectrum in a mutant of yeast cytochrome c_1 that is blocked in cleavage of the signal peptide (Yang and Trumpower, 1993).

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