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Characterization of the Chloroplast Cytochrome b_6f Complex as a Structural and Functional Dimer†

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ABSTRACT: Size analysis of the cytochrome b_6f complex by FPLC Superose-12 chromatography and Blue Native PAGE indicated a predominantly dimeric component with M_r = (1.9–2.5) × 10^5. The true dimer molecular weight including bound lipid, but not detergent, was estimated to be 2.3 × 10^5. Size and shape analysis by negative-stain single-particle electron microscopy indicated that the preparation of dimeric complexes contains a major population that has a protein cross section 40% larger than the monomer, binds more negative stain, and has a geometry with a distinct 2-fold axis of symmetry compared to the monomeric complex. The dimeric species is more stable at higher ionic strength with respect to conversion to the monomeric species. SDS–PAGE of monomer and dimer preparations indicated that both contain the four major polypeptides in approximately equal stoichiometry and also contain the *pet*G M_r 4000 subunit. One bound chlorophyll *a* per monomer, part of the bound lipid, is present in monomer and dimer. The *in vitro* electron-transport activity (decyl-PQH_2 → PC–ferricyanide) of the separated dimer was comparable to that of the isolated b_6f complex and was 4–5-fold greater than that of the monomer preparation, whose activity could be attributed to residual dimer. No difference in the properties of the dimer and monomer was detected by SDS–PAGE or redox difference spectrophotometry that could account for the difference in activities. However, the concentration of the Rieske [2Fe-2S] center was found by EPR analysis of the g_y = 1.90 signal to be lower in the monomer fraction by a factor of 3.5 relative to the dimer. The presence of active dimer at high levels in the detergent-extracted b_6f complex, the absence of activity in the monomer, and the absence of a monomer preparation that is not degraded in its spectral properties and activity suggest that the simplest inference is that the dimer is the active complex in the membrane. The possibility that cytochrome b_6f and bc_1 are primitive *trans*-membrane-signaling complexes is noted.

The cytochrome b_6f complex is one of three integral membrane protein complexes involved in electron transport in membranes that carry out oxygenic photosynthesis. The b_6f complex occupies an electrochemically central position in the noncyclic electron-transport chain, accepting electrons from the photosystem II reaction center that is associated with O_2 evolution, and donating them to the photosystem I reaction center that reduces ferredoxin and nicotinamide adenine dinucleotide phosphate ($NADP^+$).[1] The b_6f complex bears many similarities to the cytochrome bc_1 complex of the mitochondrial respiratory chain and the purple photosynthetic bacteria, in particular, its central redox position, similar prosthetic groups [two *b* hemes, the *f* (a *c*-type) heme, and the high-potential [2Fe-2S*] center], and extensive amino acid sequence identity of the cytochrome *b* and b_6 and subunit IV polypeptides (Widger *et al.*, 1984). The structure of the 252-residue lumen-side domain of cytochrome *f* has been solved at a resolution of 2.3 Å (Martinez *et al.*, 1994). A monomeric b_6f complex, including the *pet*G gene product (Haley & Bogorad, 1989), would contain 9–10 *trans*-membrane helices (Cramer *et al.*, 1991), depending on whether the [2Fe-2S*] protein contains 1 such helix (Szczepaniak *et al.*, 1991).

Understanding the mechanism of action of any protein or protein complex is likely to depend on knowledge of its oligomeric state. In the case of membrane proteins, the probability of formation of dimeric or oligomeric protein complexes is significant in the two-dimensional space of the membrane (Grasberger *et al.*, 1986).

Information bearing on the existence of dimeric states of the mitochondrial bc_1 and chloroplast b_6f complexes was previously reviewed (von Jagow & Sebald, 1980; Cramer *et al.*, 1987; O'Keefe, 1988). The first indications for the existence of a dimeric cytochrome bc_1 complex extractable from the membrane in a dimeric form came from the following: (i) studies on the cytochrome *b* of fungal (*Neurospora crassa*) mitochondria with M_r = 30 000 on SDS–PAGE and M_r = 55 000–63 000 determined by size-exclusion chromatography or ultracentrifugation (Weiss, 1976); the

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[1] Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll, cyt, cytochrome; Da, dalton(s); DBMIB, 2,5-dibromomethyl-6-isopropylbenzoquinone; DCCD, dicyclohexylcarbodiimide; EM, electron microscopy; EPR, electron paramagnetic resonance; FPLC, fast performance liquid chromatography; MEGA-9, nonanoyl-*N*-methylglucamide; M_r, relative molecular weight; MW, molecular weight; "n" and "p", electrochemically negative and positive sides of the membrane, respectively; $NADP^+$, nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PC, plastocyanin; PQ, plastoquinone; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); Q, quinone.

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question of whether the dimer was hetero- or homogeneous was eventually settled by the finding of only one gene for the cytochrome *b* in this complex (Nobrega & Tzagoloff, 1980); (ii) an approximate M_r value of 4×10^5 for the isolated bc_1 complex from beef heart mitochondria determined from sedimentation studies (von Jagow *et al.*, 1977; Weiss & Kolb, 1979) implied a predominantly dimeric bc_1 structure, as did an optical absorption and scattering analysis of the smaller complex from the thermophilic bacterium PS3 (Sone & Takagi, 1990); (iii) two-dimensional crystals of the bc_1 complex from *N. crassa* and bovine mitochondria contained a dimer in the unit cell (Leonard *et al.*, 1981).

The major suggestions and countersuggestions that have been made concerning the functional significance of a structurally dimeric cytochrome bc_1 complex are as follows: (i) Monomer–dimer transitions are involved in regulation of the respiratory rate, the dimeric form having the greater (by a factor of 2–3) activity (Nalecz & Azzi, 1985; Schmitt & Trumpower, 1990); the monomer–dimer balance *in vitro* could be shifted toward the dimer by increased ionic strength (Nalecz & Azzi, 1985), and possibly through binding of the small (M_r 16 000) acidic subunit 6 of the bc_1 complex that was proposed to be controlled by the protonmotive force (Schmitt & Trumpower, 1990). (ii) The dimeric bc_1 complex (a) has a very high (ca. 10^3 s^{-1}) rate of cytochrome *c* reductase activity in the presence of saturating amounts of lipid (Schägger *et al.*, 1990) and (b) can mediate proton translocation activity with an H^+/e ratio = 1.8 when reconstituted into liposomes at a quinone/complex ratio of 0.5 (Linke *et al.*, 1986). The latter data were interpreted in the context of an alternating "Q cycle" model with the single Q shared by the two protomers each containing two hemes *b* and an iron–sulfur center. Redistribution of the hydrophobic inhibitors between two protomers was inferred from nonlinear inhibition curves (Bechmann *et al.*, 1992). Roles for a dimeric bc_1 complex in a proton-pumping mechanism (von jagow & Sebald, 1980) and in a "double Q cycle" (De Vries *et al.*, 1983) had previously been proposed; inhibition of electron-transport activity of the bacterial photosynthetic bc_1 complex *in situ* by substoichiometric concentrations of antimycin and stigmatellin also implied a dimer (Fernandez-Velasco & Crofts, 1991). (iii) The dependence of inhibitory effects caused by the "n"- and "p"-side electron-transport inhibitors antimycin and myxathiazol on the binding of the inhibitor of proton translocation, DCCD, at a stoichiometry of 0.5/complex, and the resulting inhibition of H^+ translocation but not electron transport, led to the inference of (a) a dimeric bc_1 complex consisting of two electrically interacting monomers and (b) the possibility that the Q cycle model predicting obligatory coupling between H^+ and e^- transfer might need modification (Nieboer & Berden, 1992).

The existing data in the literature on the functional significance of the dimeric cytochrome b_6f complex are somewhat ambiguous: (i) the cytochrome b_6f complex was visualized as a particle of size sufficient for a dimer through freeze–fracture electron microscopic visualization of the complex reconstituted into liposomes (Mörschel & Staehelin, 1983); (ii) the presence of monomer and dimer forms of the b_6f complex was inferred from the presence of two bands of different, but unknown, molecular weights in a sucrose density gradient, differences in the cross-linking pattern of these two bands, and the ability of a cross-linking agent to prevent conversion of the larger form to the smaller (Chain & Malkin, 1991); (iii) a functional dimer was suggested by complete inhibition of noncyclic electron transport by 0.5 molecule per b_6f complex of the quinone analog DBMIB (Graan & Ort, 1986); the repetition of this experiment by a different laboratory yielded a different result, complete inhibition at a concentration of one DBMIB per b_6f complex (Rich *et al.*, 1991); (iv) a monomeric b_6f complex has been isolated from cyanobacteria, although no activity data have been reported (Bald *et al.*, 1992).

The present studies indicate that the cytochrome b_6f complex can be isolated from the thylakoid membrane in a predominantly dimeric form that has unique 2-fold symmetric profile when visualized by electron microscopy. The dimer has an activity comparable to the higher values previously reported for isolated b_6f complexes whose oligomeric state was not determined (Hurt & Hauska, 1981; Hauska, 1986; Black *et al.*, 1987).

MATERIALS AND METHODS

Purification of Cytochrome b_6f Complex. The complex was purified as in Black *et al.* (1987) in a procedure based on that of Hurt and Hauska (1981), except for the following: (i) the thylakoid membrane strip–wash step in 2 M NaBr was done twice; (ii) the chlorophyll concentration was adjusted to 4 mg/mL after the washing procedure; (iii) the stripped membranes were extracted in 1% sodium cholate/25 mM MEGA-9 (or 0.5% dodecyl β-D-maltoside); (iv) after addition of $(NH_4)_2SO_4$ to 0.4 M, the sample was centrifuged at 35000*g* (40 min); (v) after $(NH_4)_2SO_4$ fractionation and subsequent dialysis against 30 mM Tris–succinate (pH 7.8)/0.02% dodecyl maltoside, the complex was fractionated on a 7–30% sucrose gradient according to Hurt and Hauska (1981) and Hauska (1986); the gradient was prepared in a 9.4-mL volume containing 30 mM Tris–succinate, pH 7.8, and 0.15% dodecyl maltoside to which a 1.6-mL sample was added, using an SW-41 swinging-bucket rotor; (vi) all buffers contained protease inhibitors (0.2 mM PMSF, 2 mM benzamidine, and 2 mM ϵ-aminocaproic acid).

Size-Exclusion Chromatography. A 100-μL aliquot of purified b_6f complex (300 μg of protein) was injected onto a Superose-12 column (Pharmacia FPLC) equilibrated at 4 °C with 30 mM Tris-HCl, pH 8.0, 20 mM NaCl, and 0.15% dodecyl maltoside. The flow rate was 0.4 mL/min in most cases with the flow monitored by the absorption at 280 nm. The column was calibrated in the presence of detergent as in Suarez *et al.* (1984) with the standards throglobulin (MW = 669K), ferritin (440K), catalase (232K), γ-globulin (160K), aldolase (158K), bovine serum albumin (67K), colicin E1 (57K), and chymotrypsinogen (24K). All of these proteins are not expected to bind detergent except possibly BSA and colicin. The deviation of BSA from the linear fit to the data, as in Suarez *et al.* (1984), would be removed if it was assumed that BSA bound a micelle equivalent of detergent. The same calibration that is shown in Figure 1 was obtained in the absence of detergent using thyroglobulin, ferritin, catalase, and aldolase (data not shown).

Dimer Stability. Dimer fractions from the calibrated Superose-12 column were concentrated to 30 μM and incubated in (i) low ionic strength buffer (30 mM Tris-HCl, pH 8.0, 20 mM NaCl, and 0.15% dodecyl maltoside) or (ii) higher ionic strength buffer [100 mM Tris-HCl, pH 8.0, 100 mM $(NH_4)_2SO_4$, and 0.15% dodecyl maltoside] at 0 °C for different time intervals.

SDS–PAGE. The complex was solubilized in 50 mM Tris-HCl, pH 8.6, containing 8 M urea, 4% SDS, 10% glycerol, and 4% mercaptoethanol, and subjected to SDS–PAGE on 15% acrylamide, as in Black *et al.* (1987). The molecular

weight standards used were as follows: rabbit muscle phosphorylase *b*, 97 400; bovine serum albumin (BSA), 66 200; hen egg white ovalbumin, 45 000; bovine carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; hen egg white lysozyme, 14 400.

Western Blots. Low molecular weight proteins were separated on a 15–20% acrylamide gradient containing 8 M urea. The Western blot was carried out as described by Szczepaniak and Cramer (1990) except that the transfer was done at 138 mA (30 min) using PVDF membranes.

Blue Native Gel Electrophoresis. Blue Native PAGE was performed as described in Schägger and von Jagow (1991), except that a linear 5–16% acrylamide gradient gel with a 4% sample gel on top was used and the cathode buffer contained 0.002% Serva blue G dye, 50 mM Tricine, and 15 mM Bis-Tris, pH 7.0, at 4 °C.

Sample Preparation for Electron Microscopy. Dimer and monomer fractions were collected from the Superose-12 gel, diluted 10-fold with buffer (30 mM Tris-HCl, pH 8.0, 20 mM NaCl, and 0.15% dodecyl maltoside), and stained immediately. Negatively stained specimens were prepared by applying 2.5-μL droplets of freshly prepared cyt b_6f complex (0.01–0.005 mg/mL) to glow-discharged carbon films on 400-mesh copper grids. The grid was stained with 1% uranyl acetate solution for 30–60 s after a quick rinse with double-distilled H_2O. Electron microscopy was carried out with a Philips EM420 (Philips Electronics Instruments, Mahwah, NJ) operated at 80 kV and recorded with Kodak SO-163 film (Eastman Kodak Co., Rochester, NY) at a nominal magnification of 49000×. Micrographs exhibiting minimal astigmatism and specimen drift were digitized at 25-μm intervals (corresponding to 0.51-nm nominal sampling at the specimen) on a rotating-drum microdensitometer (Optronics Model C-4100; Optronics International Inc., Chelmsford, MA). Images of negatively stained samples were first displayed and "floated" with an average background on a 1280 × 1024 pixel raster graphics device (Model 3400; Lexidata Co., Billerica, MA) with interactive FORTRAN programs (Baker *et al.*, 1988) on a VAX/VMS 8550 minicomputer (Digital Equipment Co., Maynard, MA). The images were averaged after rotational alignment by cross-correlating their Fourier transforms. The diameters of the particles were determined from the circular averages (Olson & Baker, 1989). Digitized raw and averaged images were photographed on a graphics recorder (Model 3000; Matrix Instruments, Orangeburg, NY).

Spectrophotometry. Absolute spectra were obtained with a Uvikon 810 scanning spectrophotometer using PC-based data collector software from Research Instruments International (San Diego, CA). Room temperature chemical difference spectra were measured as described by Tae and Cramer (1992), except for the use of a Model 124 Pacific Instrument photometer run at a 1-μA setting and removal of the Balzers DT-Gruen filter. The measuring light half-bandwidth was 1.0 nm, and standard cuvette geometry was employed. For spectra at 77 K, vertical measuring beam geometry was used, and samples were dispersed in 1/1 glycerin/H_2O, 0.25 M potassium phosphate, pH 7.8.

EPR spectra were measured with a Bruker 300E spectrometer, with the sample temperature regulated using a continuous-flow cryostat, EST 900 (Oxford Instruments, Ltd.).

Activity Measurements. The electron donor decylplastoquinone (Sigma) was reduced by passing a stream of ethanol-saturated H_2 gas over a 25 mM quinone solution sealed in a capped vial in the presence of a catalytic amount of 5% "platinum on carbon" (Aldrich). The electron acceptor plastocyanin was purified according to Ellefson *et al.* (1980) except that AG-X4 resin (Bio-Rad) and a 3 × 10 cm DEAE-cellulose (DE52) column (two passages) were used, and fractions were collected with a A597/A278 ratio of >0.9 instead of >1.5. The activity was monitored by following ferricyanide reduction at 420 nm vs a 500-nm reference (differential extinction coefficient of ferricyanide at 420 vs 500 nm is 1×10^3 M^{-1} cm^{-1}) using the spectrometer described above in the dual-wavelength mode and a Princeton Applied Research (PAR) lock-in amplifier (Model 122). The PAR output was digitized with a Macintosh SE and Lab-SE I/O board (National Instruments). The rates were calculated by subtraction of the base line arising from addition of quinol alone, and by converting the differential intensity data to the differential absorbance change. The assay medium consisted of 20 mM MOPS, pH 7.0, 50 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, and 0.02% dodecyl maltoside. The concentrations (μM) of ferricyanide, decyl-PQH_2, PC, and b_6f complex were 500, 25, 5, and 0.01, respectively.

Lipid Determination. Lipids were extracted and assayed as in Szczepaniak *et al.* (1991).

RESULTS

Characterization of Dimeric Cytochrome b_6f Complex. The 4 major subunits of the cytochrome b_6f complex isolated from spinach chloroplasts are cytochrome *f* (285 residues, MW = 31 372), cytochrome b_6 (214 residues, MW = 24 038), the Rieske iron–sulfur protein (179 residues, MW = 18 922), and subunit IV (160 residues, MW = 17 444) [MW information in Widger and Cramer (1991)]. The b_6f complex can be separated into discrete fractions of different size by chromatography on a calibrated FPLC size-exclusion ("Superose-12") column. The controls for the calibration are discussed under Materials and Methods. Figure 1A shows the deconvolution of the elution profile into component bands and Figure 1B the bands tentatively associated with dimer and monomer complexes, respectively. The M_r values including bound detergent of the dimer (D) and monomer (M) components shown in Figure 1A,B, using the calibration curve of Figure 1, are approximately 240 000 and 156 000, respectively. The molecular weight of a bound micelle of dodecyl β-D-maltoside (MW = 511; micelle number, 98) would be approximately 50 000 (Dekker *et al.*, 1988). Thus, the chromatographically determined molecular weights of dimer and monomer corrected for bound detergent would be approximately 190 000 and 106 000, respectively. The molecular weight of the monomer consisting of the four major polypeptide subunits and three to four small subunits with $M_r \approx 4000$, including prosthetic groups but not bound lipid, is 105 000–110 000.

The elution profile of the monomer fraction separated from the experiment shown in Figure 1B, after a 48-h incubation at 0 °C, is shown in Figure 1C. When the dimer separated from a profile such as that of Figure 1B is incubated at 0 °C, it is slowly converted to an apparent monomer form. The elution profiles of the separated dimer determined after 24- and 120-h incubation at 0 °C at a relatively high ionic strength are shown in Figure 2A,B. The time course of the dimer → monomer conversion at high and low ionic strength was determined from the areas under the deconvoluted dimer and monomer components shown in Figure 2A,B. Approximately half of the dimer is converted to monomer under both conditions, and the half-conversion occurs with a half-time of approximately 20–30 h and 2 h, respectively (data not shown). Thus, the stability of the dimeric cytochrome b_6f complex is

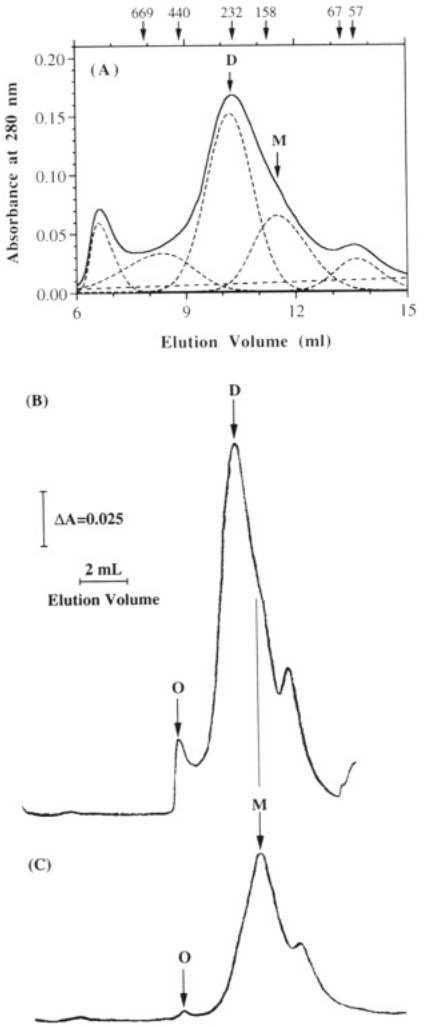

FIGURE 1: (A, B) Elution of the cytochrome b_6f complex from a calibrated FPLC size-exclusion column (Pharmacia Seperose-12 column) in the presence of 30 mM Tris-HCl, 20 mM NaCl, and 0.15% DM. Approximately 300 μg of protein was added to the column. Deconvolution of the elution profile into components is shown in (A). (C) Elution of the monomer fraction that was isolated by separating the dimer fraction from the elution profile shown in (B) and incubating for 48 h in elution buffer (above) at 0 °C. O, oligomer; D, dimer; M, monomer. Molecular mass standards are thyroglobulin (MW, kDa) (669), ferritin (440), catalase (232), γ-globulin (160), aldolase (158), bovine serum albumin (BSA, 67), colicin E1 (57), and chymotrypsinogen (24).

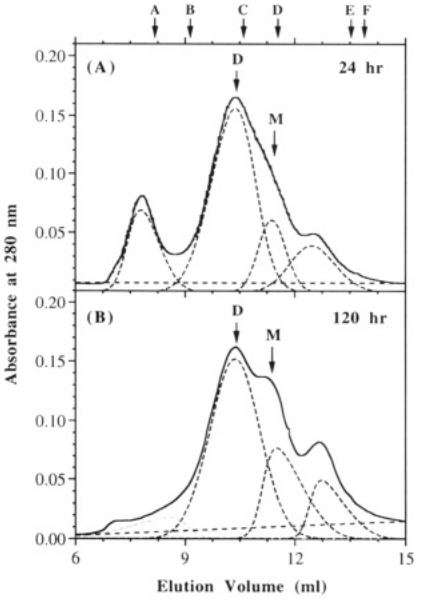

FIGURE 2: Elution profile of the dimer component isolated from a chromatographic profile such as that shown in Figure 1 after incubation at 0 °C at high [100 mM Tris-HCl/100 mM $(Na)_2SO_4$] ionic strength for (A) 24 h and (B) 120 h before application to the Superose-12 column. Dimer (D); monomer (M). Chromatography in 0.15% DM. Molecular mass standards noted as in Figure 1.

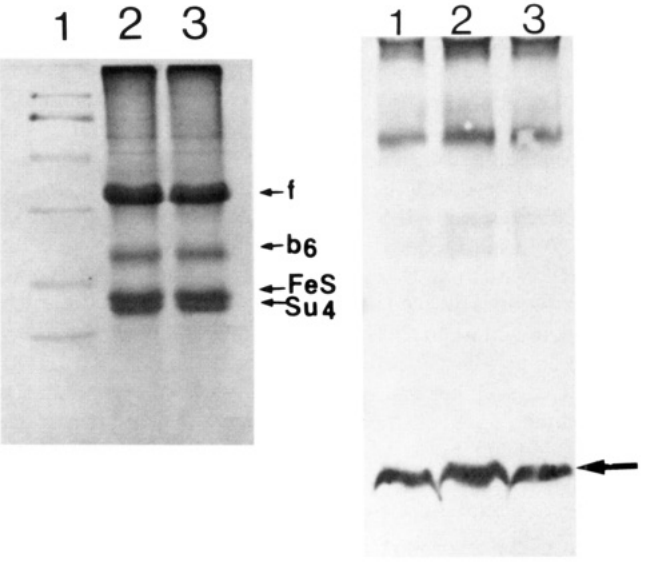

FIGURE 3: (Left panel) SDS–PAGE (15% acrylamide) of putative dimer (lane 2) and monomer (lane 3) fractions of the cytochrome b_6f complex from a Superose-12 column with M_r values of 240 000 and 160 000, respectively. Molecular mass standards (lane 1) are as described under Materials and Methods: 97.4, 66, 45, 31, 21, 14.4 kDa (Right panel) SDS–PAGE and Western blot of subunit 5 using a 15–20% acrylamide gradient for the unfractionated complex (lane 1), dimer (lane 2), and monomer (lane 3). Protein content of lanes 2 and 3, 15 μg.

increased at higher ionic strength, as had been found previously for the cytochrome bc_1 complex (Nalecz & Azzi, 1985). There is, in addition, an elution product of smaller size, $M_r \approx 50\,000$–70 000, eluting at a volume of approximately 13 mL. It is a degradation product for which the initial analysis indicated a deficiency in cyt b_6 and subunit IV (data not shown).

SDS–PAGE analysis of the dimer and monomer complexes isolated by Superose-12 FPLC chromatography indicated no significant difference in the relative polypeptide content of either the four largest polypeptides (Figure 3A, left panel) or the M_r = 4000 polypeptide (Figure 3B, right panel). Regarding the implication of preferential association of a small subunit with the dimer (Schmitt & Trumpower, 1990), there is no indication of differences in the subunit polypeptides that have been examined. No comparison has yet been made using an additional $M_r \approx 4000$ polypeptide in the complex that has recently been partially sequenced (Schmidt & Malkin, 1993). This polypeptide and one to two more small ($M_r \leq 4000$) polypeptides in the complex that have not yet been characterized are unlikely to be contaminants because they are present in the second-dimension SDS–PAGE of the complex (H. Schägger, unpublished data) separated by native PAGE (Figure 4).

The oligomeric state of the cytochrome b_6f complex was analyzed by Blue Native PAGE that has been demonstrated to separated the multisubunit membrane protein complexes of the mitochondrial respiratory chain according to their respective molecular masses with essentially no bound detergent (Schägger & von Jagow, 1991). The method is based on (i) the use of the zwitterionic 6-aminocaproic acid in the gel as a salt to improve the solubilization of membrane proteins

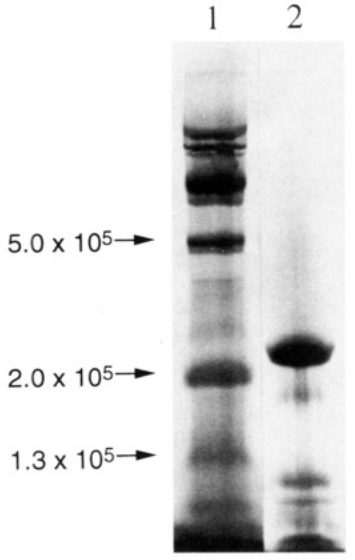

FIGURE 4: Blue Native PAGE analysis of the cytochrome b_6f complex prepared as described under Materials and Methods except for the use of 0.5% DM for extraction. Lane 1, solubilized bovine heart mitochondria; the bands near 2×10^5 and 5×10^5 molecular weight were calibrated, and identified. Lane 2, cytochrome b_6f complex. Proteins were stained in the native state during Blue Native PAGE, and the gel (5–16% acrylamide gradient) was photographed between glass plates.

that do not separate in the electrophoretic field and (ii) the use of the dye Serva blue G to impose the necessary negative charge on the electrophoresed proteins in the absence of the denaturing SDS. Blue Native PAGE of solubilized bovine heart mitochondria and of the cytochrome b_6f complex solubilized with dodecyl maltoside is shown in lanes 1 and 2, respectively, of Figure 4. It has been shown by comparison with the purified complexes that the individual respiratory electron-transport complexes can be identified in Blue Native PAGE of mitochondrial membranes (Schägger & von Jagow, 1991). The cytochrome bc_1 complex (complex III, $M_r = 5 \times 10^5$, dimer), cytochrome oxidase (complex IV, $M_r = 2 \times 10^5$, monomer), and sccinate dehydrogenase (complex II, $M_r = 1.3 \times 10^5$) are shown as standards in lane 1. The M_r values of the mitochondrial complexes were determined by the use of a large range of hydrophobic membrane proteins as standards (Schägger *et al.*, 1994). The predominant band of the b_6f complex runs with an M_r value of 250 000, above that of the M_r 200 000 cytochrome oxidase and well below the M_r 500 000 dimeric mitochondrial bc_1 complex. A small band of relatively low amplitude (<10% of the total) observed in lane 2 at an $M_r \approx 100\,000$, below the M_r 130 000 band in lane 1 associated with mitochondrial complex II, is thought to be the monomeric b_6f complex.

Electron Microscopy of Negatively Stained Dimer and Monomer Fractions. Dimer and monomer fractions isolated from the respective FPLC elution fractions (Figure 1A,B) were negatively stained and examined in a transmission electron microscope. The images of 59 dimer and 58 monomer particles observed at a magnification of 49000× were analyzed from these fields. From the dimer fields, approximately 30–40% of the particles appeared larger than particles in the monomer field, and these were selected for averaging. Two different unaveraged monomer and dimer particles are shown in Figure 5A,B and Figure 5 D,E, respectively. The averages of 58 monomer and 59 dimer particles are shown in Figure 5C,F. The appearance of these "average" particles was not affected by using different particles as the starting reference. The particles all have a protein core that is surrounded by an

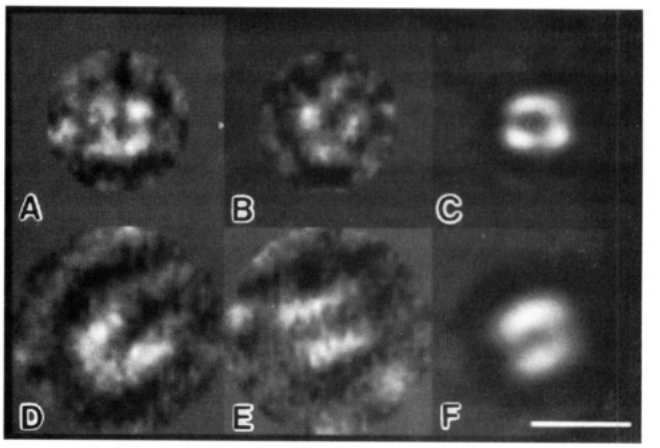

FIGURE 5: Electron micrographs of the negatively stained cyt b_6f complex at a magnification of 49000×. (A, B) Two unaveraged monomer particles. (C) Averaged image of monomer computed from 58 particle images. (D, E) Two unaveraged dimer particles. (F) Averaged image of dimer computed from 59 particle images. The average dimension of the protein central mass of the monomer and dimer is 77 ± 10 and 91 ± 9 Å, respectively (Student *t*-test parameter, 99.9%). Size bar = 10 nm.

irregular layer of negative stain and/or stain–detergent. The monomer (Figure 5C) appears to have three to four separated units of protein density, and a characteristic diameter obtained from a circular average, or calculated from the high intensity area, of 77 ± 10 Å. The corresponding dimension of the dimer which has two larger separated approximately equivalent units of density surrounded by a thicker layer of stain is 91 ± 9 Å. The Student *t*-test parameter calculated to assess the significance of the difference in these mean diameters was 99.9%, taking into account the 58 and 59 particles involved in the calculation of the circular averages.

The ratio of the protein area of dimer to monomer is 1.40. The upper limit of the molecular weight of the protein dimer was calculated to be 250K from the observed geometry and the assumption that the protein specific volume is 1.35 Å^3/Da (Richards, 1974). This value is very approximate and is an upper limit because is neglects internal cavities. A schematic explanation of the monomer and dimer perspectives is presented in Figure 6. In Figure 6B, the viewing direction of the dimer relative to the major axes of the monomer is changed, exposing a view with the cleft separating two distinct monomer subunits. A possible stacking arrangement of four elliptical subunit monomers would explain (i) the decrease in the number of symmetry units and (ii) the large volume of stain seen in the dimer (Figure 5F) compared to the monomer (Figure 5C). Single-particle negative-stain EM analysis has also been reported for the cytochrome b_6f complex from the cyanobacterium *Synechocystis* PCC 6803, from which a monomeric structure was inferred (Bald *et al.*, 1992).

Visible Absorption Spectra. The absolute spectrum of the dimeric cytochrome b_6f complex in the wavelength range 400–700 nm is shown in Figure 7A. The absorption peak of the bound chlorophyll in the dimer and monomer preparation is at 669.0 and 669.5 nm, respectively, indicative of chlorophyll *a*. The bound chlorophyll/b_6f complex ratio in the dimer, using an extinction coefficient of 20 mM^{-1} cm^{-1} (554–540 nm) for cytochrome *f* (Cramer & Whitmarsh, 1977) and 82 mM^{-1} cm^{-1} for chlorophyll *a* in organic solvent (Hoff & Amesz, 1991), was approximately (0.8–0.95)/1, implying one bound chlorophyll *a* molecule per monomeric unit. A similar value was calculated for the monomer (data not shown). The ratio of the Soret band to red peaks was greater by approximately 30% in the unfractionated complex (data not shown), indi-

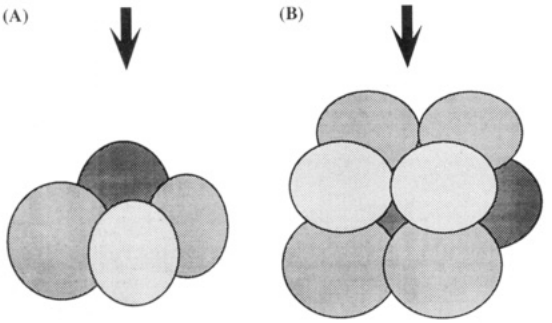

FIGURE 6: Schematic representation of the suggested orientation of the monomer (A) and dimer (B) b_6f complex in the negative-stain images. Two different viewing directions are shown (arrows) relative to the major axes of a putative four-subunit monomer and the homodimer.

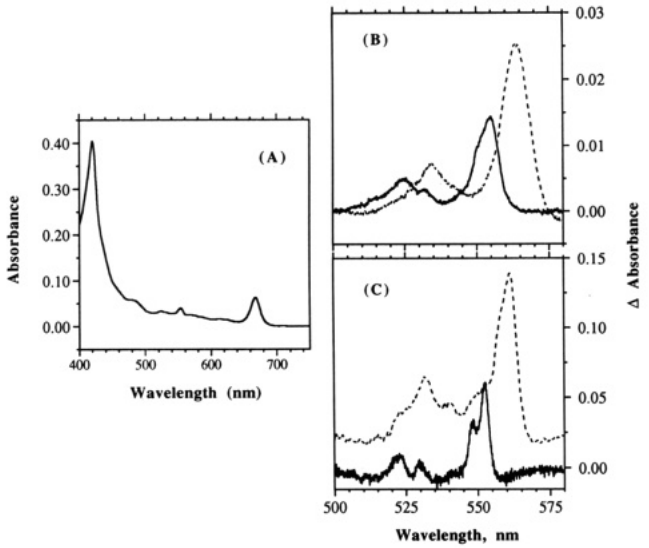

FIGURE 7: Absorbance spectra (no redox agent added) of the dimeric d_6f complex. Absolute visible absorbance spectrum (A) and redox difference spectra at room temperature (B) and 77 K (C): menadiol (0.1 mM; added after 1 mM hydroquinone) minus ferricyanide (0.1 mM) (dashed curve) and dithionite (ca. 2 mM) minus menadiol (solid curve) difference spectra. The spectral peaks (half-bandwidth in parentheses) of cytochrome f (menadiol – ferricyanide) in the dimer at room temperature and 77 K, respectively, are 554.4 (9.1) and 552.3, 548.1 nm; peaks (half-bandwidths) of cyt b_6 (dithionite – menadiol) in the dimer are 563.8 (10.8) and 561.0 (7.4) nm.

cating that some free chlorophyll was lost during FPLC purification of the dimer and monomer. However, there was no more than 5% loss of the chlorophyll during further FPLC passage of the complex, indicating that the binding to the dimer was not adventitious. The presence of chlorophyll in the isolated b_6f complex can be seen in the spectrum obtained for the original preparation of Hurt and Hauska (1981). The cytochrome b_6f complex isolated from *Synechocystis* PCC 6803, inferred to be monomeric, was also found to contain a single bound oriented chlorophyll *a* (Bald *et al.*, 1992).

In Vitro Activity of Dimeric and Monomeric b_6f Complex. The *in vitro* electron-transfer activity of the dimeric b_6f (Figure 8A) from the donor decyl-PQH_2 to PC–ferricyanide as acceptor is shown in Figure 8 to be 4.4 times that for the monomeric b_6f (Figure 8B). The activities of the dimer and the monomer were 77 and 18 electrons $(\text{cyt } f)^{-1}\ s^{-1}$, respectively. This ratio is large enough that the activity of the monomer can be attributed to residual dimer in the preparation, and the monomer can be considered inactive (see Spectra of Rieske Iron-Sulfur Protein). The activity of the dimer is also comparable to the rate measured for the unfractionated cyt b_6f complex, 82 electrons $(\text{cyt } f)^{-1}\ s^{-1}$ (data not shown). The

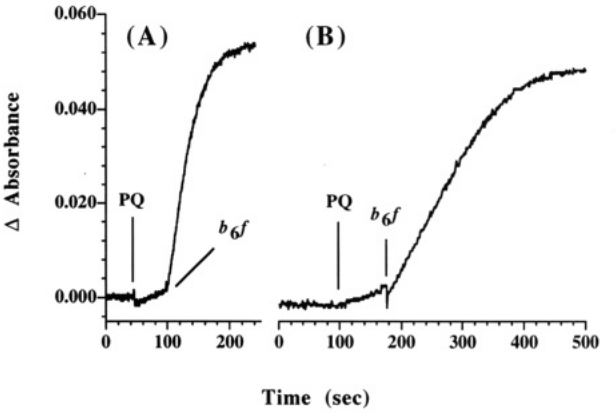

FIGURE 8: *In vitro* electron-transport activity from decyl-PQH_2 (to PC–ferricyanide catalyzed by dimer (A) and monomer (B) fractions separated according to the elution profile in Figure 2. The specific activities were 77 (n = 2, individual measurements, 82 and 72) and 18 (n = 2, individual measurements, 17 and 19) electrons $(\text{cyt } f)^{-1}\ s^{-1}$ for dimer and monomer, respectively. Activity was measured by the differential absorbance change at 420 vs 500 nm as described under Materials and Methods. The activities of dimer and monomer with ferricyanide alone as the electron acceptor were 48 ± 3 (n = 5) and 9 ± 0.6 (n = 3) electrons $(\text{cyt } f)^{-1}\ s^{-1}$. Assay medium: 20 mM MOPS, pH 7.0, 50 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, and 0.02% DM. Concentrations of ferricyanide, decyl-PQH_2, PC, and b_6f complex (μM): 500, 25, 5, and 0.01, respectively.

Q_p-site inhibitor stigmatellin (1 μM) completely inhibited the reaction (data not shown). The ferricyanide in these experiments primarily acts to oxidize the PC after its reduction by b_6f, thereby reducing the amount of PC consumed during each trial. The ferricyanide does, however, participate in the direct oxidation of b_6f but at a specific rate 50 times slower than that of PC. Ratios of dimer to monomer activity obtained using only ferricyanide or PC as the electron acceptor (data not shown) are similar to those obtained with PC and ferricyanide.

Redox Difference Spectra. Because the electron-transfer rate of the monomer was very low compared to that of the dimer and all major polypeptides were present in the monomer (Figure 3A), redox difference spectra of the cytochromes (Figure 7B,C) and the Rieske iron–sulfur center (Figure 9A–D) were measured.

Redox difference spectra of the separated dimeric cyt b_6f complex are shown at room temperature (Figure 7B) and 77 K (Figure 7C). The reduced α-band peak of cytochrome f is 554.5 nm at room temperature, and 552.3 nm (548.3 nm, peak of split band) at 77 K. The ratio of heme b to heme f was close to 2 to 1. The corresponding reduced α-band peaks for the cytochrome b_6 hemes are at 563.8 nm at room temperature and 561.0 nm at 77 K. Spectrophotometric data obtained for the unfractionated complex and monomer were similar to those of the dimer (data not shown).

Spectra of the Rieske Iron–Sulfur Protein. Figure 9 shows EPR spectra of the Rieske iron–sulfur proteins in the cytochrome b_6f complex in the ascorbate-reduced (A, B) and the dithionite-reduced (C, D) state, respectively. Spectra A and C were obtained from the Rieske protein in the monomeric form of the b_6f complex, while spectra B and D were obtained from the dimeric form at almost equivalent protein concentrations. Both preparations exhibit Rieske spectra with $g_{x,y,z}$ = 2.03, 1.90, ~1.75, and a line shape typical for a Rieske cluster in the complex which contains fully reduced pool quinone. As clearly seen in this figure, the spin concentrations of the EPR-active ascorbate-reduced cluster is much less in the monomeric form than in the dimeric form [7.5 μM (A) versus 26.6 μM (B)]. The spin concentrations did not change

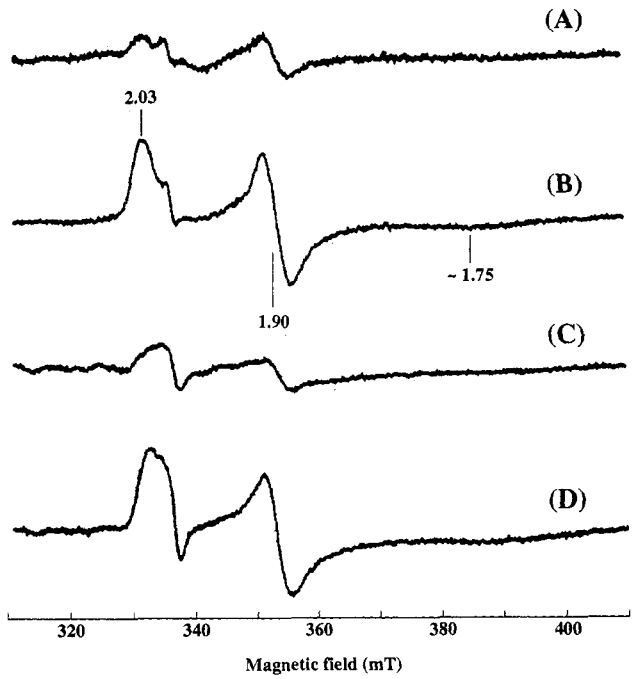

FIGURE 9: EPR spectra of the reduced Rieske iron–sulfur cluster in the monomer (A and C) and dimer (B and D) forms of the cytochrome b_6f complex. Both monomer and dimer preparations were dissolved in 30 mM Tris-HCl, pH 7.8, 4 °C, containing 20 mM NaCl and 0.15% DM and were reduced either with 5 mM sodium ascorbate in the presence of 20 μM PMS (A, B) or with 5 mM dithionite in the presence of 20 μM phenosafranine and 5 μM pyocyanine (C, D). Concentration of cyt b_6f complex, 26.5 and 30 μM cytochrome f for the monomeric and dimeric forms. EPR parameters: microwave power, 4 mW; modulation amplitude, 1.01 mT; time constant, 163.8 ms; sweep rate, 17.9 mT/min; microwave frequency, 9.44 GHz; sample temperature, 17 K. Spin quantitations were conducted at 250-μW microwave power, keeping all other EPR conditions unchanged, and utilizing 500 μM Cu-EDTA as a standard.

upon reduction with dithionite in the presence of appropriate redox dyes, showing that the smaller spin intensity of the Rieske iron–sulfur cluster in the monomer form is not a consequence of a lowered midpoint redox potential compared to that of the cluster in the dimer form. When the content of EPR-active Rieske iron–sulfur center is normalized to the protein concentration, it is concluded that the dimer complex, with 1.0 spin from the iron–sulfur cluster per cyt f, contains 3.5 times more EPR-active Rieske cluster than does the monomer. These observations imply a deficiency in the content of functionally intact Rieske iron–sulfur cluster in the monomer compared to the dimer that almost quantitatively matches the loss in electron-transfer activity.

DISCUSSION

Characterization of the Dimer and Monomer of the Cytochrome b_6f Complex. (A) Molecular Weight. The M_r value of the dimer determined by FPLC molecular sieve chromatography and Blue Native PAGE was approximately 190 000 (molecular sieve, corrected for bound detergent)–250 000 (native gel). This compares to a molecular weight for the four large and three to four small subunits in a lipid-free monomer, including prosthetic groups, of approximately 110 000. Lipid analysis of DM-extracted complex showed the content of lipid plus detergent to be 1.0 and 1.1 mg/mg of protein for dimer and monomer, respectively, of which >90% is detergent (data not shown). There appeared to be approximately 10 bound lipid molecules in the monomer, including 1 chlorophyll, and approximately 20 in the dimer including 2 chlorophylls. The actual molecular weights including bound lipid, but not detergent, would then be approximately 115 000 and 230 000, respectively.

(B) Electron Microscopy. The dimer particle had a larger and different appearance compared to the monomer when viewed by electron microscopy using negatively stained samples (Figure 5). An interpretation of the EM data requires that the orientation of the monomer and the dimer should be different (Figure 6). With this reservation, the dimer was found to have a profile with 40% larger area in projection and to bind substantially more stain (*n.b.*: if the particles were perfectly spherical, the diameter and area ratios would be 1.26 and 1.59). An upper limit estimate of 250 000 for the molecular weight of the dimer with a mean dimension of 91 Å was based on the observed geometry neglecting internal cavivites. The most obvious speculation of the nature of the three to four subunits seen in the monomer is that they correspond to the four major subunits, cyt f, cyt b_6, [2Fe-2S*] protein, and subunit IV (suIV), of the complex. This idea could be tested by immunolabeling of the individual subunits. The central cavity or depression in the monomer image and the cleft in the 2-fold symmetric image of the dimer invite the speculation that this is a channellike structure. In any case, the image of the putative dimer with a 2-fold axis of pseudosymmetry (Figure 5F) is consistent with the concept that it is a true dimer.

Function of Dimer and Lack of Function of Monomer. The electron-transfer activity of approximately 80 electrons (cyt f^{-1} s^{-1} of the isolated dimeric particle is proof that this dimer preparation is active. The 4–5-fold lower activity of the monomer can be almost quantitatively attributed to incompetence of the monomer arising from loss of an active Rieske iron–sulfur center. The activity in the monomer preparation is low enough that it can reasonably be attributed to residual dimer in the monomer fraction collected from the FPLC. It is inferred that the monomer fraction is inactive, that is is a somewhat degraded preparation, and that there is at present no evidence associating activity with monomeric b_6f complex.

The topic of the functional role of dimeric or oligomeric structures is an important one in the field of membrane proteins. There is widespread agreement that the mitochondrial cytochrome bc_1 complex can function as a structural and functional dimer (von Jagow & Sebald, 1980; Linke *et al.*, 1986; Schmitt & Trumpower, 1990; Schägger & von Jagow, 1991; Nieboer & Berden, 1992), although there is disagreement over whether the dimeric form is obligatory (Schägger & von Jagow, 1991) or whether monomer and dimer forms coexist and their interconversion is important in the regulation and mechanism of electron transport (Schmitt & Trumpower, 1990). Although evidence for a structural dimer (Mörschel & Staehelin, 1983) and for two centrifugally separable forms, presumably monomer and dimer (Chain & Malkin, 1991), has been presented, it has also been argued on the basis of inhibitor stoichiometry that the b_6f complex can function as a monomer (Rich *et al.*, 1991). No activity measurements were made in the study of Chain and Malkin (1991). The data in the present study imply that dimeric cyt b_6f complex is present in the membrane and is active.

Functional Significance of Dimeric Cytochrome b_6f Complex. In the case of the cytochrome b_6f complex, the question of whether an unmodified Q cycle model suffices to describe electron/proton transfer has been raised (Girvin & Cramer, 1984; Cramer *et al.*, 1987; Furbacher *et al.*, 1989), and

counterarguments have also been put forward (Rich *et al.*, 1991; Kramer & Crofts, 1992, 1993). Without presenting the details of this discussion, we note some aspects of a functional dimer that could be relevant to the question of mechanism and particularly the experimental problem of why the amplitude of the light flash-induced reduction of cytochrome b_6 is often well below unity even in the presence of inhibitor: (i) the finding of two bound quinones at the p-side of the cytochrome bc_1 complex of *Rb. capsulatus* (Ding *et al.*, 1992) might be considered in the context of a dimeric complex; (ii) quinol oxidation by two [2Fe-2S*] centers of the dimer could allow direct formation of the fully oxidized quinone, thus removing an obligatory role of the semiquinone intermediate, or regulating its presence through a monomer–dimer equilibrium (Cramer *et al.*, 1987); (iii) the existence of the dimeric b_6f unit would provide an explanation for the subunitary amplitude of single light flash-induced reduction of cyt b_6 that is often observed (Furbacher *et al.*, 1989; Rich *et al.*, 1991). If the dimer were obligatory for function, then the maximum stoichiometry of heme reduction/center would be 0.5 assuming that the probability of electron transfer is identical in the two promoter units.

Finally, it is of interest that the current concepts for ligand-mediated *trans*-membrane signaling include a ligand-induced monomer → dimer transition (Canals, 1992). However, the actual nature of the signal that crosses the membrane is not understood (Bormann & Engelman, 1992). The cytochrome b_6f and bc_1 complexes may be primitive *trans*-membrane-signaling structures. This possibility is suggested by the association of the b_6f complex with a kinase whose activity is affected by the action of *trans*-side inhibitors (Gal *et al.*, 1990). The *trans*-membrane proton translocation associated with the cytochrome complexes may be related to the mechanism of *trans*-membrane signaling.

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