Electron Transfer from the Rieske Iron-Sulfur Protein (ISP) to Cytochrome f in Vitro

IS A GUIDED TRAJECTORY OF THE ISP NECESSARY FOR COMPETENT DOCKING?

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The time course of electron transfer in vitro between soluble domains of the Rieske iron-sulfur protein (ISP) and cytochrome f subunits of the cytochrome b_{6}f complex of oxygenic photosynthesis was measured by stopped-flow mixing. The domains were derived from Chlamydomonas reinhardtii and expressed in Escherichia coli. The expressed 142-residue soluble ISP apoprotein was reconstituted with the [2Fe-2S] cluster. The first-order rate constant, k_{f}^{ISP-o} = 1.5 \times 10^{6} \text{M}^{-1} \text{s}^{-1}, for ISP to cytochrome f electron transfer was <10^{-2} of the rate constant at low ionic strength, k_{f}^{PC}(>200 \times 10^{6} \text{M}^{-1} \text{s}^{-1}), for the reduction of plastocyanin by cytochrome f, and 1/30 of k_{f}^{PC} at the ionic strength estimated for the thylakoid interior. In contrast to k_{f}^{PC}, k_{f}^{ISP-o} was independent of pH and ionic strength, implying no significant role of electrostatic interactions. Effective pK values of 6.2 and 8.3, respectively, of oxidized and reduced ISP were derived from the pH dependence of the amplitude of cytochrome f reduction. The first-order rate constant, K_{f}^{ISP-o}, predicted from k_{f}^{ISP-o} is ~10 and ~150 times smaller than the millisecond and microsecond phases of cytochrome f reduction observed in vivo. It is proposed that in the absence of electrostatic guidance, a productive docking geometry for fast electron transfer is imposed by the guided trajectory of the ISP extrinsic domain. The requirement of a specific electrically neutral docking configuration for ISP electron transfer is consistent with structure data for the related cytochrome bc_{1} complex.

The luminal and intramembrane domains of the cytochrome b_{6}f complex of oxygenic photosynthesis contain four redox centers: cytochrome f with one covalently bound b-type heme, cytochrome b with two noncovalently bound b-type hemes, and one [2Fe-2S] cluster in the Rieske iron-sulfur protein (1–3). The electron transfer pathway, plastoquinol → Rieske ISP → cytochrome f → plastocyanin or cytochrome c_{6} → photosystem I on the lumen (p-side) of the membrane, comprises the high potential electron transport chain of the plastoquinol oxidase, whereas electron transfer between the two b-hemes, heme b_{1} → heme b_{o}, to a putative n-side bound quinone defines the low potential chain. Absorption of a photon and charge separation in photosystem I causes the oxidation of cytochrome f by plastocyanin (t_{1/2} = 200 \mu s, k_{f}^{PC} = 3000 \text{s}^{-1}) (4–7) after which the cytochrome is re-reduced by the Rieske iron-sulfur protein, which is usually poised in the reduced state in the dark, with half-time, t_{1/2} = 5–6 ms (first-order rate constant, k_{f}^{ISP-o} = 250–150 \text{s}^{-1}). Only about half of the chemical content of cytochrome f is observed to turn over with this half-time after a short, 5-μs flash (8–10). The other half has been inferred to be reduced too rapidly to be observed (10), with a rate constant, ~10^{-1} \text{s}^{-1}, presumably similar to the fast phase of reduction of cytochrome c_{1} in the bc_{1} complex (11).

Structure studies on the related cytochrome bc_{1} complex from bovine and avian mitochondria showed the extrinsic domain of the ISP and the [2Fe-2S] cluster itself in well separated conformations, which depend on the crystal form and the presence of inhibitors bound to the complex (12, 13). The two extreme positions of the [2Fe-2S] cluster, separated by ~16 Å, are (i) proximal to the site (Q_{o}) of the bound ubiquinol inhibitor analogue, e.g. stigmatellin, and the cytochrome b polypeptide; and (ii) proximal to cytochrome c_{1} via the heme propionate of the π-bonded heme (12). Neither of the individual conformations would allow the ISP to serve as both an electron acceptor for quinol oxidation and an electron donor to cytochrome c_{1} (14). The net 16-Å translation of the [2Fe-2S] cluster occurs as a result of a rotation of the globular extrinsic domain by ~57° around an axis that includes the 9-residue peptide that links the globular extrinsic and transmembrane domains (12, 15–17). At least one additional stable intermediate position along this rotational trajectory has been defined (15). The inference that electron transfer mediated by the ISP in the cytochrome bc_{1} complex occurs via its rotational-translational movement between donor and acceptor sites, dependent upon flexibility of the linker region, has been supported by mutation-function analysis of the linker peptide segment (16, 18, 19).

Given the homologies: (a) between the cytochrome b_{6}/subunit IV polypeptides and mitochondrial cytochrome b measured at low resolution (20), (b) the hydrophathy and histidine ligation acid; PC, plastocyanin; PSI, photosystem one; p-side, electrochemically positive side of the membrane; Q_{o}, quinone binding site on the electrochemically positive side of the membrane; TRICINE, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; E_{m}, midpoint redox potential.

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identities predicted from the respective gene sequences (21), (c) the cluster-binding domains of the Rieske ISP (22), and (d) in addition the flexible nature of the polyglycine putative linker region in the ISP of the b$_f$ complex (22), it is inferred that the ISP in the b$_f$ complex also carries out its electron transfer function by a large-scale rotation-translation between donor-acceptor sites. This inference is supported by orientation changes of the principal g values of the [2Fe-2S] system in the isolated b$_f$ complex induced by the p-side quinone analogue inhibitor, 2,5-dihydro-3-methyl-6-isopropylbenzoquinone (23) or metal ions (24), and the systematic effect of increased ambient viscosity on the rate of reduction of cytochromes f and b$_6$ in thylakoid membranes (25).

The 3–5 ms half-time for the reduction of cytochrome f by the Rieske ISP is the rate-limiting step of the b$_f$ complex, and of oxygenic photosynthesis. The events included in the observable reduction of cytochrome f include: (i) electron transfer from plastoquinol to the ISP; (ii) release of the ISP from the quinol-proximal binding site; (iii) tethered movement of the ISP from its quinol-proximal site to a site close to the cytochrome f heme, as described above; (iv) electron transfer from the docked ISP to cytochrome f.

Stopped-flow and equilibrium measurements were employed to follow the kinetics and pH dependence in vitro of the docking and electron transfer reaction of soluble redox-active fragments, 142 and 252 residues, respectively, of the Rieske ISP and cytochrome f. These data show that electron transfer in solution between these partners cannot rely on electrostatically guided docking, as occurs with plastocyanin/cytochrome f; the second-order rate constant obtained in vitro predicts a first-order rate constant in vivo that is substantially smaller than the observed values.

MATERIALS AND METHODS

Plasmid Construction—Plasmid pOSH37d was constructed to produce the 142-residue Chloramydomonas reinhardtii chloroplast Rieske iron-sulfur fragment, CRN37d (142 residues, deletion of 37 N-terminal residues). A segment of the PetC gene was amplified from Chloramydomonas RNA by reverse transcriptase-PCR and inserted into plasmid pET22a (Novagen, Madison, WI). This plasmid (pOSH37d or pTRXCRN37d) carries a fused open reading frame for thioredoxin, a 6-histidine tag for affinity purification, two thrombin cleavage sites, and the Rieske CRN37d ISP under control of a T7 RNA polymerase promoter.

Expression of the Truncated Fragment of the Rieske Iron-Sulfur Protein—Reconstitution of the 2Fe-2S Cluster and Protein Purification

Procedures for Chloramydomonas ISP were based on those developed previously for cyano bacterial ISP.2 Echerichia coli AD494(DE3) (Novagen) carrying pOSH37d was grown to early exponential phase (40–60 Klett units, Klett-Summmerson colorimeter) in LB medium supplemented with 150 mg/ml ampicillin, 1 mg/ml FeSO$_4$, and 2 mg/ml cysteine typically at 25 °C. Isopropyl-p-o-thiogalactopyranoside was added to 0.1 mg/ml and incubation was continued overnight. Harvested cells were suspended in 50 ml Tris-HCl buffer at pH 8.2. Urea was added to 8 M and the cells were broken by three passages through a French pressure cell at 20,000 p.s.i. and 4 °C. The ISP fusion protein in the crude supernatant fraction was reconstituted with iron-sulfur clusters as described in Refs. 26 and 27 but with the detergents and dialysis steps omitted. Fusion protein was purified directly from the reconstitution mixture (after dilution of urea to 0.8 M) by fast protein liquid chromatography (Amersham Biosciences) and either His-Bind (Novagen) or DEAE-Sepharose Fast Flow (Amersham Biosciences) resins. The latter, which sometimes gave cleaner separation, was used for the current work. The crude, reconstituted fusion protein preparation was loaded onto the column in 20 mM Tris-HCl, pH 8.3, buffer and eluted with a 0–1.0 M gradient of NaCl in the same buffer. The fusion protein, which eluted at about 300 mM NaCl, was concentrated by ultrafiltration (Amicon YM10 membrane) to several milligrams/ml and cleaved overnight with thrombin (0.2 units/mg of protein) at room temperature. Cleavage yielded a 13.9-kDa His-tagged thioredoxin fragment, a 3.9-kDa linker fragment, and the 14.9-kDa CRN37d ISP. The ISP was purified by passage over nickel-nitrilotriacetic acid His-Bind Superflow resin (Novagen) followed by dialfiltration (YM-10 membrane) to a concentration of about 1.7 mg/ml (107 µM). The protein concentration of the ISP was estimated by its absorbance at 280 nm and predicted extinction coefficient of 3.17 × 10$^4$ M$^{-1}$ cm$^{-1}$ (28).

EPR Spectroscopy of the Rieske ISP—EPR spectra were obtained on a Varian E-4 X-band spectrometer as described previously (26) at −15 K, 5 mW microwave power, 10 G modulation amplitude, 0.128-ns time constant, and 100 KHz modulation frequency. ISP samples were found to be nearly fully reduced as prepared because the addition of ascorbate produced minimal increases in signal amplitude. The spin concentration of ISP [2Fe-2S] clusters was estimated by double integration of the EPR signal against a copper-perchlorate standard (29).

Expression of a Soluble Fragment of Cytochrome f in E. coli—A 252-residue redox-active fragment of wild type cytochrome f was overproduced in E. coli as described in Ref. 7. The cytochrome f construct was co-transformed with pEC86 that carries the cassette of cytochrome c maturation genes (30) into strain MV1190. The cells were grown semianerobically at 37 °C for 20–24 h. The cells were harvested and broken by osmotic shock with 20% sucrose in 30 mM Tris-HCl, pH 7.5. Crude cytochrome f in the supernatant was passed through a DE-52 ion-exchange column. Fractions containing cytochrome f were then pooled, concentrated, and passed through a Sephadex G-100 size exclusion column. Cytochrome f fractions with A$_{554}$/A$_{280}$ > 0.7 were then passed through a hydroxypatite column, fractions with A$_{554}$/A$_{280}$ > 0.9 were collected.

Purification of Plastocyanin from C. reinhardtii—Plastocyanin was isolated and purified from wild-type C. reinhardtii cells through ammonium sulfate precipitation as described in Ref. 6.

Reaction of ISP with Cytochrome f—The rate of reduction of oxidized cytochrome f by the soluble ISP fragment from C. reinhardtii was determined by following changes in the cytochrome absorbance at 421 nm in a stopped-flow spectrophotometer (Applied Photophysics SX.185MV) at room temperature. Cytochrome f was oxidized by potassium ferricyanide and the excess oxidant was removed by filtration through a Centric 10 filtration unit. Second-order rate constants were obtained from a set of pseudo-first order rate measurements, in which this rate was measured as a function of the concentration of cytochrome f, 10-, 15-, and 20-fold greater than that of the ISP. Conditions for which the concentration of ISP was in excess of that of cytochrome f yielded the same results. The reaction of oxidized cytochrome f and ISP under equilibrium conditions was carried out by mixing the two proteins and then scanning the spectrum of cytochrome f from 400 to 580 nm in a Cary 3 (Varian) spectrophotometer. All titrations were performed with 0.1 M NaOH. The spectrum of cytochrome f at each pH was recorded in a Cary 3 spectrophotometer. The amount of cytochrome f reduced was calculated from the amplitude of the a (554 nm) difference band at each pH using an extinction coefficient of 2.6 × 10$^4$ M$^{-1}$ cm$^{-1}$ (31).

RESULTS

Overproduction and Purification of the Rieske ISP—The soluble fragment of the Chloramydomonas chloroplast Rieske protein was expressed in E. coli as a chimeric thioredoxin/Rieske ISP fusion protein and reconstituted in vitro with iron and sulfide to regenerate the 2Fe-2S cluster. SDS-PAGE of the purified fusion protein is shown (Fig. 1A). Thrombin cleavage, passage over nickel-nitrilotriacetic acid resin, and ultrafiltration generated the 14.9-kDa Rieske ISP (Fig. 1B). Restoration of typical Rieske 2Fe-2S clusters to the expressed ISP is demonstrated by the low temperature EPR spectrum (g$_1$ = 1.75, g$_2$ = 1.90, g$_3$ = 2.03), characteristic of the high potential Rieske [2Fe-2S] protein (Fig. 1C). The 142-residue ISP (CRN37d) carries two residues (Gly and Ser) of the thrombin cleavage site in front of the flexible hinge region starting at residue Phe-38. Based on the estimation of protein concentration and spin quantification of the EPR signal, the cluster content of the reconstituted ISP was ~65%. The ascorbate-reduced minus ferricyanide-oxidized chemical difference spectrum for the ISP
has broad positive and negative peaks at 400 and 476 nm (data not shown) with relatively weak extinction coefficients. This implies that: (a) the more narrow absorbance bands of cytochrome f, with much higher extinction coefficients, would be more useful for optical assay of electron transfer between the pair; and (b) the interference from ISP absorbance would be negligible (~0.4% in the presence of equimolar amounts of the two proteins) in the assay of cytochrome f reduction measured using the Soret band of the latter (Fig. 2).

In Vitro Reaction between C. reinhardtii ISP and Cytochrome f—The soluble ISP was able to transfer an electron to oxidized cytochrome f, as shown by the decrease in absorbance of cytochrome f at 410 nm and the increase in its absorbance at 421 nm (Fig. 2). The spectrum of oxidized cytochrome f is defined by a sharp Soret peak at 410 nm whereas its a band around 554 nm is featureless. The increase in amplitude of the Soret peak at 421 nm associated with its reduction by the ISP is accompanied by the appearance of a distinct a-band at 554 nm. Redox reactions of cytochrome f were observed by following changes in the Soret band at 410 or 421 nm, rather than the a-band whose extinction coefficient is ~1/3 that of the Soret band.

Stopped-flow measurements of the reaction between reduced Rieske ISP and oxidized cytochrome f yielded a second-order rate constant, \( k_{\text{red}}^{\text{ISP}} \approx 1.5 \times 10^8 \text{M}^{-1} \text{s}^{-1} \). Typical traces of the kinetics of cytochrome f reduction at pH 5.6 and 7.5 are shown in Fig. 3A and summarized in Table I. Under the same reaction conditions, the rate of electron transfer from cytochrome f to PC is more rapid, with \( k_{\text{red}}^{\text{PC}} \approx 200 \times 10^8 \text{M}^{-1} \text{s}^{-1} \), respectively, at pH 7, and 0.01 and 0.2 mM ionic strength (6, 32). No variation in the kinetic reaction constants associated with the Soret peak were observed between pH 7.5 and 5.6. Although there was no observable pH dependence for \( k_{\text{red}}^{\text{ISP}} \), the amplitude of cytochrome f reduction was larger at pH 7.5 than at 5.6 (Fig. 3A). The kinetics of cytochrome f reduction by ISP was also measured as a function of wavelength to generate a "stopped-flow spectrum" of the reaction (Fig. 3B). The stopped-flow spectrum peaks at 421 nm, and is virtually identical to the Soret band of the reduced minus oxidized chemical difference spectrum, indicating that the kinetics observed in the stopped-flow experiment were specifically those associated with cytochrome f reduction.

pH Titration of the Reaction of ISP and Cytochrome f—The \( E_m \) of cytochrome f is pH-independent between pH 4 and 8 (33), whereas the 139-residue ISP isolated from spinach chloroplasts has a pH-dependent \( E_m^{\text{ISP}} \Delta E_m(pH) = -30 \text{ mV/pH} \) (34). A larger pH dependence of the \( E_m^{\text{ISP}} \Delta E_m(pH) = -60 \text{ mV/pH} \) was obtained for the ISP [2Fe-2S] cluster in isolated cytochrome b,f complexes from spinach (35). In either case, \( \Delta E_m \) increases, and the amount of cytochrome f reduced by ISP in the reaction, ISP(red) + cytochrome f(ox) → ISP(ox) + cytochrome f(red), increases with increasing pH (Figs. 2 and 4A). A plot of the amplitude of cytochrome f reduction by an equimolar quantity of ISP (Fig. 4A) reflects the pH dependence of the \( E_m \) of the ISP (34, 35). The pH dependence of the extent of cytochrome f reduction by the ISP at each pH was identical when measured in the a- and Soret bands of cytochrome f. Cytochrome f is ~50

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**Fig. 1.** SDS-PAGE and EPR spectrum of the Chlamydomonas Rieske fragment. Panels A and B display the SDS-PAGE of the overproduced, purified, and reconstituted TRXCRN37d fusion protein, and the Rieske ISP after thrombin cleavage and separation from the fusion protein. Lanes 1 in both panels show molecular mass standards (14.4, 21.5, 31, and 45 kDa). Panel C shows an EPR spectrum of the purified and reduced ISP (protein concentration, 107 μM) obtained as described under "Materials and Methods." The characteristic g values of the Rieske 2Fe-2S cluster are shown.

**Fig. 2.** Reduction of cytochrome f by the Rieske ISP in vitro. The bold trace is a spectrum of oxidized cytochrome f alone. The thin solid and dashed traces are spectra of cytochrome f after reaction with the Rieske ISP at pH 8.5 and 4.1, respectively. Reaction mixtures contained 0.5 μM reduced ISP, 0.5 μM oxidized cytochrome f, 0.5 mM EDTA, 10 mM each of succinate, MES, HEPES, TRICINE, and CHES buffers.

**Fig. 3.** Kinetics of cytochrome f reduction by the Rieske ISP in vitro. A, reactions were performed at pH 5.6 (solid line) and pH 7.5 (dashed line) at room temperature in a stopped-flow spectrophotometer (see "Materials and Methods"). Cytochrome f reduction was monitored by absorbance changes at 421 nm. The "control" trace contained only buffer and oxidized cytochrome f. B, stopped-flow spectrum (open circles) of the cytochrome f/ISP reaction, obtained by following the reaction kinetics at several wavelengths around 421 nm, superimposed on the chemical difference spectrum of reduced-oxidized cytochrome f. Reaction mixtures contained 0.25 μM reduced ISP, 0.25 μM oxidized cytochrome f, 0.5 mM EDTA, and 10 mM HEPES-NaOH at pH 5.6 or 7.5.
In vitro oxidation-reduction of cytochrome f

- Table I

| ISP → Cyt f | Cyt f → PC | ISP → Cyt f | Cyt f → PC | ISP → Cyt cyt f |
|-------------|------------|-------------|------------|----------------|
| 150–250     | ~2,400–3,000 | 0.01        | ~1.5      | >200          |
| 0.20        | ~1.5       | 50          |           | 16,000 (23), 250 (1/3) |

* Rates constants for oxidation-reduction of cytochrome f.
* Rates constants obtained from stopped-flow data in the present study.
* Rates constants obtained from measurements in isolated cytochrome bc complexes; 2/3 and 1/3 of the amplitude of the cytochrome c reduction is associated with the high and low rate constants (11).
* I(ionic strength).
* Rate constant determined from in vitro reaction of cytochrome f and PC initiated by flash photolysis of flavin-semiquinone (32, 47).

**Fig. 4.** pH dependence of the reaction between oxidized cytochrome f and reduced Rieske ISP. A, amplitude of cytochrome f reduction by the ISP as a function of pH, shown as the amplitude of the α-band, 554 nm, of reduced cytochrome f. The reaction was initiated at pH 4.1; pH values were adjusted as described under “Materials and Methods.” B, pH dependence of the E*m of the ISP. The E*m at each pH was obtained from the data in A and the known E*m of cytochrome f that is constant at ~370 mV from pH 4 to 8.5. The solid line is a fit to E*m = E*m,low pH – 59 log((Kox + H+)/2|Kred + H+)), where Kox and Kred are acid dissociation equilibrium constants for the oxidized and reduced species, respectively. Although two pK values of 7.6 and 9.2 were inferred for the oxidized form of the ISP from bovine heart mitochondria (36), the fit to the present data does not require a fit to two pK values. The slope of the curve in the pH-dependent region from pH 6 to 8.5 is ~40 mV/pH, in agreement with the approximately ~30 mV/PbH obtained from optical titration in the visible spectrum (34), but smaller than the ~80 mV/PbH obtained for isolated b complex by EPR titration of the midpoint potential (35). Titration of the pH dependence of the E*m of the ISP from cytochrome bc complexes, using the weak 500-nm band of the circular dichroism spectrum, yielded slopes of about ~120 mV/PbH at pH > 8 and ~60 mV/PbH below pH 8 (36, 37).

**DISCUSSION**

**Does the kISP-f Measured In Vitro Account for the Observed Rate of Cytochrome f Reduction in S itu and in Vivo?**—To calculate the first-order rate constant in vitro, kISP-f measured in vitro, one must estimate the intramembrane constant concentration of the ISP as follows: (i) kISP-f (PC) in the 0.2 M solution environment estimated for the thylakoid lumen (38) is 50 x 10^9 M^-1 s^-1 (6), (ii) the observed first-order rate constant kISP-f (PC) ~2500–4000 s^-1 implies a luminal PC concentration of 6 x 10^-5 M. If there are 4 PC per cytochrome f (or ISP) (39), then the ISP concentration in vivo is ~1.5 x 10^-13 M. Using the measured kISP-f (PC) of 1.5 x 10^-6 M^-1 s^-1, an in vitro first-order rate constant of cytochrome f reduction, kISP-f, of 20 s^-1 is obtained. This value of kISP-f calculated from kISP-f (PC) is smaller than the observed kISP-f ~150–250 s^-1 for the slow phase of cytochrome f reduction. An additional rate constant that includes a microsecond rate of cytochrome f reduction is required to describe the reduction kinetics of the total complement of cytochrome f in situ or in vivo.

The Two Kinetic Components of Cytochrome f Reduction in Situ and in Vivo:—The ISP and cytochrome f components of the high-potential (E*m > 0.3 V) chain of the cytochrome b complex are initially reduced before oxidation of PSI by a short (microsecond) light flash. This results in an observable oxidation of ~50% (1:1600 chlorophyll) of the total chemical content of cytochrome f (1:800 chlorophyll) via plastocyanin with a kISP-f ~2700 s^-1 (Fig. 5A), and reduction with kISP-f ~150–250 s^-1. The missing 50% of the amplitude of the ISP oxidation-reduction can be accounted for if an additional microsecond kinetic component, kISP-f, is similar to that observed in the reduction of cytochrome c (11), is included in describing the reduction of cytochrome f (Fig. 5A). The kinetics of cytochrome f oxidation-reduction can be described as the change in absorbance, ΔA(t), according to: ΔA(t) = ΔAmax[(1 – exp(−k1ISP-f, t)) + 0.5exp(−k1fast, t) + 0.5exp(−k1slow, t)], where k1ISP-f is the first-order rate constant for cytochrome f oxidation, k1fast and k1slow are rate constants for cytochrome f reduction, and ΔAmax is the total change in absorbance associated with full oxidation of cytochrome f. As shown in Fig. 5A, rapid

and ~95% reduced at pH 4.1 and 8.5, respectively, as seen by the amplitude of the α- and Soret band peaks (Figs. 2 and 4A). The pK values for the reaction is ~6.2, which is close to the value previously measured by titration of the visible spectrum of the 139-residue ISP isolated from the spinach b complex (34), but lower than the pK values of 7.6–7.8 determined for the ISP of the b complex by EPR at cryogenic temperature (35).

**pH Dependence of the Midpoint Potential of the ISP**—The midpoint potential of the ISP at each pH point in Fig. 4B could be determined from the known amount of oxidized and reduced cytochrome f, and from the known midpoint potential of cytochrome f (E*m = 370 mV), which is pH-independent from pH 4 to 8. A plot of E*m versus pH for the ISP is shown (Fig. 4B). pK values of 6.2 and 8.2 for the oxidized and reduced forms, respectively, of the ISP were obtained by least squares fit to the

**formula**, E*m = E*m,low pH – 59 log((Kox + H+)/2|Kred + H+)), where Kox and Kred are acid dissociation equilibrium constants for the oxidized and reduced species, respectively. Although two pK values of 7.6 and 9.2 were inferred for the oxidized form of the ISP from bovine heart mitochondria (36), the fit to the present data does not require a fit to two pK values.

The slope of the curve in the pH-dependent region from pH 6 to 8.5 is ~40 mV/pH, in agreement with the approximately ~30 mV/PbH obtained from optical titration in the visible spectrum (34), but smaller than the ~80 mV/PbH obtained for isolated b complex by EPR titration of the midpoint potential (35). Titration of the pH dependence of the E*m of the ISP from cytochrome bc complexes, using the weak 500-nm band of the circular dichroism spectrum, yielded slopes of about ~120 mV/PbH at pH > 8 and ~60 mV/PbH below pH 8 (36, 37).

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re-reduction of 50% of the cytochrome $f$ prevents observation of the full oxidation of the total chemical content of cytochrome $f$, in this case $k_{1}^{f}(PC) = 2700 \text{ s}^{-1}$ and $k_{1,slow}^{ISP-f} = 3200 \text{ s}^{-1}$. To observe full oxidation, the rate of cytochrome $f$ oxidation should be much faster (Fig. 5B), or its rate of reduction, $k_{1,slow}^{ISP-f}$, much slower (Fig. 5C) than the observed rates.

**ISP and Plastocyanin Utilize Different Mechanisms to Dock to Cytochrome $f$**—The slow electron transfer between ISP and cytochrome $f$ in solution that is independent of pH and ionic strength shows that the ISP and cytochrome $f$ are not able to find a competent docking site through electrostatic guidance in solution. Considering the regions of prominent electrostatic potential on the surfaces of plastocyanin and the soluble domains of cytochrome $f$ and the ISP (Fig. 6, top), it is expected that ISP, like plastocyanin, would be able to utilize electrostatic guidance. However, the prominent negative patch on the surface of the ISP lies away from the cluster so that even if electrostatic interactions are utilized to dock the proteins, the resulting complex would be unproductive.

**Hypotheses to Explain the Small $k_{1}^{ISP-f}$**—The second-order rate constant in vitro, $k_{2}^{ISP-f} = 1.5 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}$ predicts a rate constant for cytochrome $f$ reduction that is slower than the observed $k_{1,slow}^{ISP-f} = 240 \text{ s}^{-1}$ and $k_{1,fast}^{ISP-f} = 3200 \text{ s}^{-1}$ by $10$ and $150\times$, respectively. There are two obvious hypotheses that can explain the small value of $k_{2}^{ISP-f}$ that is ineffective at docking in vitro between ISP and cytochrome $f$: (i) alteration or damage to the soluble cytochrome $f$ or ISP fragments. The possibility of small but critical structural changes cannot be excluded. However, the soluble ISP has a normal optical spectrum and $E_{m}(7)$, and rapidly transfers electrons to PC with kinetics that are dependent upon ionic strength and the presence of specific basic residues. (ii) Docking and electron transfer in vitro are inefficient because of the absence of electrostatic or structural guidance. It is inferred that the flexible membrane-embedded ISP tether provides constraints that assure formation of a docked configuration of the ISP-cytochrome $f$ complex that allows more efficient electron transfer.

It can be seen that the mitochondrial ISP uses the same surface domain to dock in proximity to (i) the cytochrome $c_1$ heme (Fig. 7, A and B), and (ii) the quinol $Q_0$ (or quinone analogue inhibitor) binding site (Fig. 7C). The active face of the ISP around the [2Fe-2S] cluster is electrically neutral in both the $b_{c_1}$ (15) and $b_{sf}$ (22) complexes (Fig. 6, bottom). The absence of any electrostatic influence on the interaction between the ISP and cytochrome $f$ in vitro implies that a specific electrically neutral docking geometry may be required for fast ISP-cytochrome $f$ electron transfer. The use of the productive docking configuration, without wasteful searching of coordinate space for unproductive docking geometries, is imposed by the guided trajectory of the [2Fe-2S] cluster in the extrinsic domain of the ISP, which rotates about the flexible interfacial linker region (16).

$E_{m}$ and $pK_{ox}$—The $pK_{ox} = 6.2–6.5$ of the isolated ISP from *C. reinhardtii* (Fig. 4B, above), or from spinach chloroplasts determined by room temperature optical difference spectra (34), is significantly more acidic than that (7.6–7.9) determined for the Rieske protein or a similar ISP from the bc complex in mitochondria and bacterial photosynthesis (>7.0). On the other hand, the difference of 1–1.5 pH units between the $pK_{ox}$ value of the chloroplast (ambient pH ~ 6) (40) and mitochondrial/bacterial (ambient pH ~ 7.0) ISP agrees with the pH difference between the ambient environments in the two membrane systems, and the requirement that the ISP be able to cycle be-
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FIG. 7. Docking of Rieske ISP and cytochrome c₆ A. ISP is shown docked to cytochrome c₁ in the bovine mitochondrial cytochrome bc₁ complex (15). ISP is shown in its 2 extreme positions: B, proximal to cytochrome c₁ (15), and C, proximal to the Qₐ-site (‘b’ position) (12). The [2Fe-2S] cluster of the ISP is shown as orange spheres. The figures were drawn with MOLSCRIPT (51) and rendered with Raster3D (52).

together protonated and deprotonated states. For the latter purpose, the pKₐ should be close to the value of the ambient pH. The pKₐ of the ISP is critical for charge-transfer reactions in the bc or bf complexes. (i) The bifurcated transfer of electrons from quinol to the ISP and to the low potential cytochrome b is believed to be initiated by deprotonation of quinol at the Qₐ site. Quinol deprotonation has been proposed to occur via one of the histidine ligands of the 2Fe-2S, as shown by the structure of the mitochondrial cytochrome bc₁ complex, one of the oxygens of stigmatellin is within H-bonding distance to His-161 of the ISP (12, 13). If the first H⁺ from quinol is indeed transferred to one of the histidine ligands of the ISP, this histidine should have a different proton state depending on the reoxid state of the ISP. The values of pKₐ = 6.2 and pKᵣᵩ = 8.3 determined in vitro raise a question about the protonation state of the ISP in vivo. At neutral pH, about 14% of the oxidized protein, or 95% of the reduced protein would be protonated at pH 6.5 (40). The reduced ISP is more than 95% protonated in this pH range, whereas the oxidized protein is about 30% protonated at pH 6.5. This consideration implies that the ISP is deprotonated only after it has been oxidized by cytochrome f.

These results for the pKₐ of the ISP of the bf complex are complicated by the finding that the pKₐ of the chloroplast ISP was >7.0 when it was measured by titration of its gₛ EPR signal at 17 K. Furthermore, the pKₐ for the ISP in the intact bf complex determined by low temperature EPR was 7.6–7.8 (35). Some difference in structure between the native Rieske protein and the ISP, affecting the location relative to the [2Fe-2S] cluster of charged residues or dipole elements, is indicated by the limiting Eₛₐ of the ISP at acid pH being 50 mV more positive than that of native ISP (34).

Rate-determining Step for Charge Transfer through the Cytochrome bf Complex—The pH dependence of the rates of reduction of cytochromes b and f in the cytochrome bf complex (41, 42), and kinetic isotope effects (43), suggest the possibility of a rate-limitation because of proton-coupled electron transfer (7). From the observed decrease in turnover rate in site-directed mutants of the ISP of the cytochrome bc₁ complex that associated with a decreased ISP Eₛₐ (44, 45), it was inferred that the quinol-ISP electron transfer step is rate-limiting in the bc₁ complex. A slow phase of cytochrome c₆ reduction, Eₛₐ(ISP) = 250 to 180 s⁻¹ and 1000 s⁻¹, respectively, observed in photoactivated electron transfer using isolated mitochondrial and bacterial bc₁ complexes, was attributed to the quinol-ISP electron transfer step (11). The fast phase of the photoactivated cytochrome c₁ reduction, Eₛₐ(ISP) = 16,000–60,000 s⁻¹ (11), was associated with electron transfer from ISP docked to cytochrome c₁, implying that electron transfer from docked ISP to cytochrome c₁ or f (see above) is also not rate-limiting.

The constrained and guided motion of the ISP can be considered as a candidate for the rate-limiting step. In the case of the bc₁ complex, it has been argued that the ISP motion is not rate-limiting (19). This is also implied by the identical rates of reduction of cytochrome c₁ and b in the photoactivated mitochondrial bc₁ complex (11). However, in the cytochrome bf complex, the slow phase of reduction of cytochromes f and bₛ were shown to be inversely proportional to the ambient lumen viscosity, implying that the tethered motion of the ISP is rate-limiting (25).

Regulation and Control of ISP Rotation—The existence of the reversible rotational motion of the ISP in cytochrome bc complexes is presumably under control of redox events in the complex, and may involve interaction of the monomeric units of the dimeric complex (46). The fact that the observed turnover of cytochrome f involves ~50% fast and ~50% slow phases suggests a state of the bf complex in which ISP is bound to cytochrome f in one monomer of the complex and bound to the quinol-proximal site in the other. However, details of the regulation/control mechanism are obscure at present.

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