Movement of the Rieske Iron−Sulfur Protein in the *p*-Side Bulk Aqueous Phase: Effect of Lumenal Viscosity on Redox Reactions of the Cytochrome b_6f Complex[†]

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ABSTRACT: Based on the atomic structures of the mitochondrial cytochrome bc_1 complex, it has been proposed that the soluble domain of the [2Fe-2S] Rieske iron−sulfur protein (ISP) must rotate by ca. 60° and translate through an appreciable distance between two binding sites, proximal to cytochrome c_1 and to the lumen-side quinol binding site. Such motional freedom implies that the electron-transfer rate should be affected by the lumenal viscosity. The flash-induced oxidation of cytochrome *f*, the chloroplast analogue of cytochrome c_1, was found to be inhibited reversibly by increased lumenal viscosity, as was the subsequent reduction of both cytochrome b_6 and cytochrome *f*. The rates of these three redox reactions correlated inversely with lumenal viscosity over a viscosity range of 1−10 cP. Reduction of cytochrome b_6 and cytochrome *f* was not concerted. The rate of cytochrome *f* reduction was observed to be approximately half that of cytochrome b_6 regardless of the actual viscosity, implying that the path length traversed by the ISP in reduction of cytochrome *f* is twice that of cytochrome b_6. This suggests that upon initiation of electron transfer by a light flash, cytochrome b_6 reduction requires movement of reduced ISP from an initial position predominantly proximal to cytochrome *f*, apparently favored by the reduced ISP, to the quinol binding site at which the oxidant-induced reduction of cytochrome b_6 is initiated. Subsequent reduction of cytochrome *f* requires the additional movement of the ISP back to a site proximal to cytochrome *f*. There is no discernible viscosity dependence for cytochrome b_6 reduction under oxidizing conditions, presumably because the oxidized ISP preferentially binds proximal to the quinone binding niche. The dependence of the cytochrome redox reaction on ambient viscosity implies that the tethered diffusional motion of the ISP is part of the rate limitation for charge transfer through the b_6f complex.

The cytochrome b_6f complex of chloroplasts and cyanobacteria is functionally situated between the two photosystems of oxygenic photosynthesis. Its integral membrane protein subunits, cytochrome b_6 and subunit IV, are related structurally, and through a common evolutionary origin to the mitochondrial and bacterial counterpart, cytochrome *b*, in the cytochrome bc_1 complex (*1*). A central feature of charge transfer through the bc_1 complex is oxidant-induced reduction (*2, 3*), which occurs in the bifurcated oxidation of the bound quinol by the high and the low potential electron-transfer chain. Upon one-electron oxidation of ubi- or plastoquinol by the high potential chain, in which the Rieske iron−sulfur protein (ISP)[1] and cytochrome *f* or cytochrome c_1 are the first two acceptors, the resultant semiquinone has a sufficiently negative redox potential to reduce heme b_P, the first acceptor of the low potential chain. The mechanism of quinol oxidation by the high potential chain is a central problem in this model (e.g., see refs *4*−*12*). Partly because its visible absorbance spectrum is weak and broad, the role of the iron−sulfur protein in the mechanism has been investigated less well than that of cytochromes c_1 and *c* in mitochondria and photosynthetic bacteria, and of cytochrome *f* in oxygenic photosynthesis.

New information on the function of the ISP in the mitochondrial bc_1 complex has been provided by atomic structures of the bc_1 complex (*13*−*16*). The iron−sulfur cluster in the soluble domain of the ISP has been found in two or three different locations separated by 10−15 Å, close to either cytochrome c_1 or the quinol binding site (*14, 15, 17*), or in an intermediate position between these sites (*16*). The distance of the [2Fe-2S] cluster from the *p*-side cytochrome *b*−heme Fe and the cytochrome c_1−heme Fe, respectively, is approximately 35 and 17−21 Å in the c_1 proximal position, and 26 and 31 Å when the ISP is proximal to the quinone binding niche. In the latter position of the cluster, the 31 Å distance from its electron acceptor is too large to allow electron transfer in the appropriate 0.01−1 ms time range (*18, 19*), leading to the inference that the ISP soluble domain can move back and forth between the quinol and cytochrome c_1 binding sites (*14*−*16*). Evidence for this "mobile ISP" model was provided by the demonstration that shortening or stiffening through mutagenic substitution of the "neck region" of the ISP, which connects the soluble domain of the ISP to its transmembrane helix, could severely

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[1] Abbreviations: cP, centipoise; chl, chlorophyll; cyt, cytochrome; η, viscosity (units: cP); ISP, iron−sulfur protein; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; Q_N/Q_P, quinone-binding site on the electrochemically negative/positive side of the b_6f complex.

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impair or even block the activity of the bc_1 complex (*8*). It was also reported that the ISP of the chloroplast b_6f complex has two different locations, with the position proximal to the Q_P binding site favored in the presence of DBMIB (*12*). However, although the existence of discrete binding positions for the ISP is well established in the bc_1 complex from X-ray structure analysis and in the b_6f complex from EPR analysis of the paramagnetic [2Fe-2S] cluster of the ISP in oriented membranes (*12*), direct evidence for movement of the ISP during electron transport is lacking.

It is shown in the present study that the flash-induced kinetics of cytochrome *f* oxidation, and the subsequent reduction of cytochrome b_6 and cytochrome *f*, are strongly inhibited by increased lumenal viscosity, with the degree of inhibition proportional to the increase in viscosity. The selective inhibitory effect of ambient viscosity on electron-transfer reactions in situ that involve diffusible carriers, in contrast to those that occur within a complex, has been demonstrated in the viscosity-dependent and -independent slow and fast phases of P_{700}^+ reduction (*20*). For cytochrome *f* oxidation, the inhibition can be explained by the hindered movement of plastocyanin, which is known to be a diffusible electron carrier (*21*, *22*). Because there also is a simple proportionality between the inhibition of the reduction of cytochrome b_6 and cytochrome *f* and increased viscosity, this is most readily explained by viscosogenically inhibited diffusion of the soluble domain of the ISP between a site proximal to cytochrome *f* and one near the Q_P binding site.

MATERIALS AND METHODS

Thylakoid Isolation. Thylakoids were isolated from young spinach leaves bought from a commercial source. All steps of the isolation process were carried out at 4 °C. A total of 30−60 young leaves were macerated for 7−8 s with 200 mL of buffer A (300 mM sucrose, 30 mM KCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 50 mM MES/KOH, pH 6.1). The homogenate was pressed through 4 layers of cheesecloth and 1 layer of nylon mesh (pore width, 100 μm), and the resulting filtrate was centrifuged for 5 min at 4000*g*. The sediment was resuspended in 20 mL of buffer C (300 mM sucrose, 30 mM KCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, and 50 mM HEPES/KOH, pH 7.6) and centrifuged again at 3000*g* (2 min) before resuspension in 2−3 mL of buffer C. Where indicated, sucrose was exchanged for glucose in both homogenization and suspension buffers. Directly before measurement, thylakoid membranes were suspended in various amounts of a buffer containing 50 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES/KOH, pH 7.6, to rupture intact chloroplast envelopes. After the sample was stirred (≥30 s), the final volume of 2 mL was obtained by adding complementary amounts of a buffer that contained either 80% glycerol, 60% sucrose, or 48% glucose, thus establishing the desired viscosity.

Flash Kinetic Spectroscopy. Measurements were conducted at room temperature (20−22 °C) with thylakoids (40 μg of chl mL^{-1}) to which 250 μM duroquinol (prepared as described in ref *23*), 0.2 μM nigericin, and 1 μM nonactin had been added. NQNO, a quinone analogue that is believed to act as a Q_N-site inhibitor (*24*−*26*), was routinely added at a concentration of 0.35 μM in all measurements. For the experiments shown in Figure 6, duroquinol was omitted, and continuous far-red background illumination (60 μE m^{-2} s^{-1}; Balzers 712 nm interference filter) was applied to establish oxidizing conditions.

Data Acquisition. The kinetics of cytochrome b_6 and *f* absorbance changes were measured as fractional transmittance changes, $\Delta I/I$, at 564 nm relative to a 575 nm reference, and at 554 nm relative to (542 nm + 575 nm)/2, respectively. Rate constants and amplitudes for the cytochrome redox reactions were fit by simulation of a linear equation of first-order processes. For cytochrome *f*, a biphasic reduction or an "overshoot" at low viscosity, i.e., a small, additional oxidation spike of small amplitude and very fast subsequent reduction, were occasionally observed. In all cases, the half-time for the major kinetic component of the reduction (>80% of the amplitude) is provided. For cytochrome b_6 reduction, a small lag phase usually was observed at high viscosity. This lag time was always much shorter than the half-time for cytochrome b_6 reduction and seemed to correlate with the time course of cytochrome *f* oxidation. The viscosity values of the various solutions were obtained from (*27*).

Micro-Hematocrit Measurements. Thylakoids were washed with a buffer containing 5 mM $MgCl_2$ and 5 mM HEPES/pH 7.6 and, after centrifugation, stored in 100 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, and 20 mM HEPES/KOH. The thylakoids were uncoupled with nigericin and nonactin and diluted to 1.7 mg of chl mL^{-1} with buffers containing various amounts of glycerol, sucrose, or glucose as viscosogenic agents.

RESULTS

Viscosity Effects on Cytochrome b_6f Redox Reactions. The flash-induced redox kinetics of cytochrome *f* and cytochrome b_6 in thylakoids are shown with characteristic half-times for cytochrome *f* oxidation (ca. 150 μs) and reduction (4 ms), and cytochrome b_6 reduction (1.6 ms) (Figure 1A). These half-times are close to established values (e.g., refs *22*, *26*, *28*−*31*). All three redox reactions are slowed considerably by addition of glycerol. The oxidation rates of cytochrome *f*, and reduction of cytochromes b_6 and *f* can be seen to be inhibited by factors between 2.3 and 3 in the presence of 32% w/w (ca. 3.7 M) glycerol (Figure 1A,B). At 40% w/w (ca. 4.8 M) glycerol, the retardation factors increased to 3.4−3.8 (Figure 1A,C). Even within single samples, there were significant variations in the degree of inhibition. However, it is shown below that, on average, the inhibition factor was the same for all three redox reactions for any given glycerol concentration (see Figures 3A and 5).

The half-time of cytochrome *f* oxidation, ca. 150 μs in the absence of viscosogenic agents, is shown as a function of the concentration of such reagents as glycerol, sucrose, and glucose, over a wide range of concentrations (Figure 2). The relationship between half-time and concentration is similar for sucrose and glucose, but it can be seen that comparable inhibition of cytochrome *f* oxidation by glycerol requires much higher concentrations. Furthermore, the observed relationship is highly nonlinear, with increasingly effective inhibition at higher concentrations. While the half-time of cytochrome *f* oxidation at 4 M glycerol (ca. 350 μs) is inhibited by a factor of slightly more than 2 as compared to control measurements (no glycerol, 150 μs), the rate is retarded by a factor of 10 at 7.5 M glycerol.

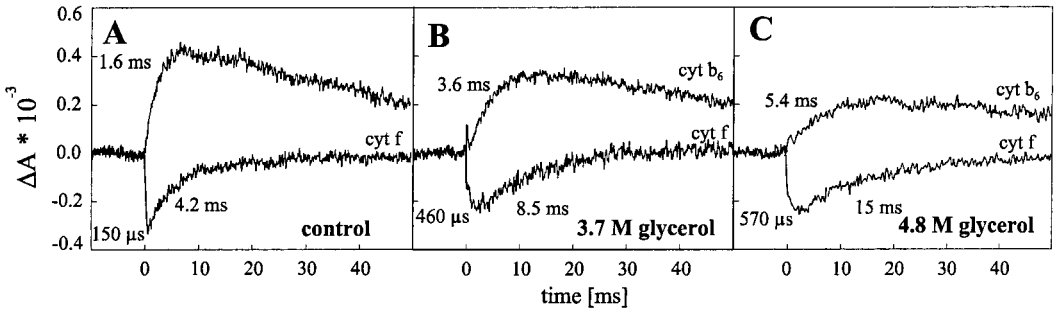

FIGURE 1: Flash-induced redox reactions of cytochromes b_6 and f in thylakoids at different concentrations of glycerol. Fresh thylakoids in the presence of 1 μM nonactin, 0.2 μM nigericin, 0.35 μM NQNO, 200 μM methyl viologen, and 250 μM duroquinol were subjected to short saturating flashes, 36 averages per wavelength (0.2 Hz). Half-times were calculated by assuming monophasic first-order kinetics for both reduction and oxidation for both cytochromes. (A) Standard suspension buffer; (B) with 32%; and (C) with 40% (w/w) glycerol.

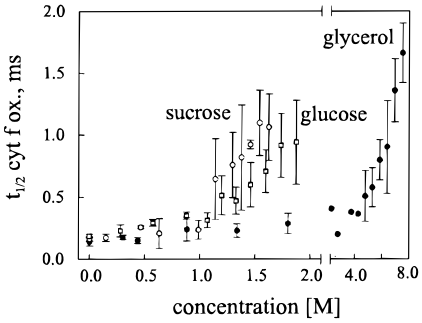

FIGURE 2: Half-time of cytochrome f oxidation as a function of osmolarity in the presence of glycerol, sucrose, or glucose. Experimental conditions otherwise as in Figure 1. Glycerol, closed circles; sucrose, open circles; glucose, squares; each with standard deviation determined from 4 to 7 measurements.

The observed kinetic inhibition was essentially fully reversible with all three viscosogenic agents; however, while the inhibitory effect of the saccharides was reversed within seconds upon dilution, reversal of the inhibitory effect of glycerol required centrifugation of the prediluted thylakoid suspension, followed by long incubation times (ca. 1 h) of the resuspended membranes. The basis for the slow reversibility by glycerol is discussed below.

The data shown in Figure 2 are plotted in Figure 3 as a function of ambient viscosity. There is a linear relationship between viscosity and the half-time of cytochrome f oxidation which is different for each of the three tested viscosogenic agents. With glycerol (Figure 3A), the slope of the linear regression line (152 μs·cP^{-1}) equals the average half-time [$t_{1/2} = 149 \pm 10$ μs ($n = 14$)] for cytochrome f oxidation at $\eta = 1$ cP (no glycerol). In other words, the extrapolated regression line passes very close to the (x/y) origin of the graph, indicative of a proportional relationship between the half-time of cytochrome f oxidation and viscosity. With sucrose as viscosogenic agent (circles in Figure 3B), the relative slope of the linear regression line (115 μs·cP^{-1}) was only ca. 3/4 that of glycerol. With glucose (squares in Figure 3B), inhibition of cytochrome f oxidation by increased viscosity was also linear, but the slope was 378 μs·cP^{-1}, much larger than with glycerol or sucrose. No significant difference could be observed between thylakoids that were isolated with, and stored in, sucrose (filled squares) or glucose buffers (open squares) before measurement.

Differential Effects of Viscosogenic Reagents: Micro-Hematocrit Experiments. The thylakoid volume decreased in the presence of increasing concentrations of sucrose or glucose, with the volume change inversely proportional to the external saccharide concentration (Figure 4). Glycerol, on the other hand, had no discernible osmotic effect at all, with the thylakoid volume independent of the glycerol concentration. This is in accordance with the demonstration that glycerol penetrates the thylakoid membrane within a few seconds (*32*). Consequently, it was found that addition of

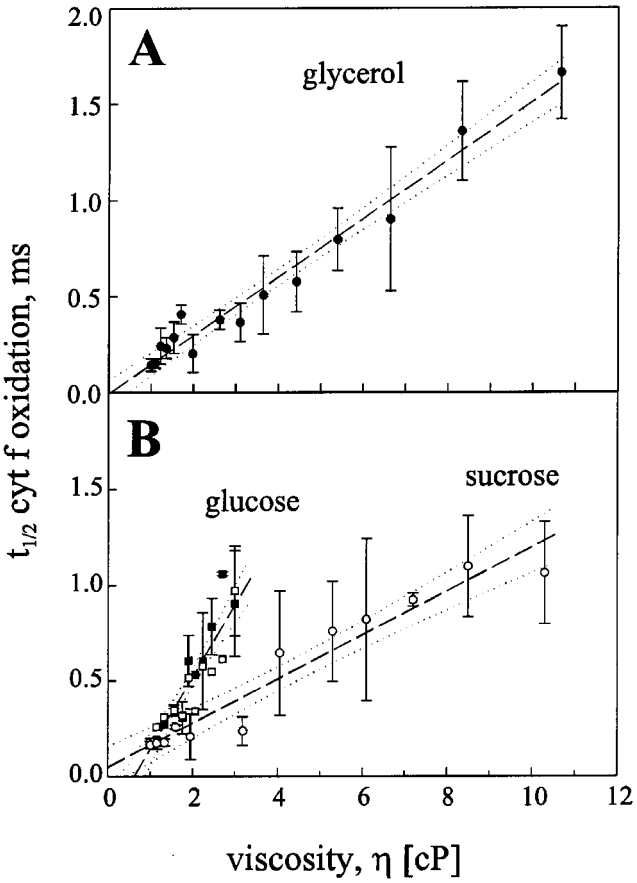

FIGURE 3: Half-time of cytochrome f oxidation as a function of viscosity for different viscosogenic solutions: (A) glycerol, (B) sucrose and glucose. Experimental conditions as in Figure 2. The linear $t_{1/2}/\eta$ regression fit to the data, represented by dashed lines, and 95% confidence intervals, indicated by the dotted lines, were as follows: (A) glycerol, $t_{1/2} = 0.15\eta - 0.01$, with 3.1, 6.6, and 10.7 cP corresponding to 4.3, 6.4, and 7.5 M, respectively; (B) sucrose (circles), $t_{1/2} = 0.12\eta + 0.05$, with 3.2, 6.1, and 10.3 cP corresponding to 1.0, 1.4, and 1.6 M; and glucose (squares), $t_{1/2} = 0.38\eta - 0.24$, with 2.1 and 3.0 cP corresponding to 1.3 and 1.9 M. With glucose, measurements were conducted with thylakoids isolated either with sucrose buffer (closed squares) or with glucose buffer (open squares).

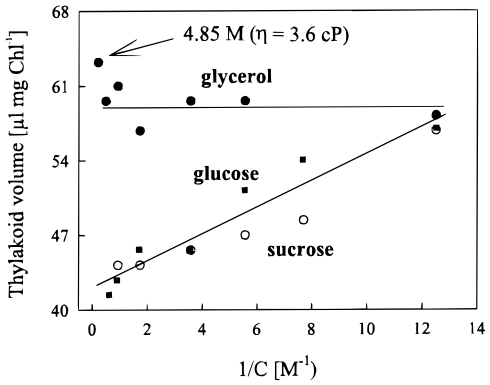

FIGURE 4: Dependence of thylakoid volume on osmolarity. Volume determined by micro-hematocrit method (volumetric centrifugation) as described under Materials and Methods. Thylakoids were incubated for 3 min in different concentrations of glycerol (closed circles), sucrose (open circles), and glucose (squares), respectively, at a concentration of 3.7 mg of chl mL^{-1}.

glycerol to the thylakoid membranes caused no detectable effect on the 90° scattering measured at 780 nm (data not shown), while sucrose and glucose induced a substantial increase in scattered light that relaxed very slowly, with half-times of ca. 1 h and 20−30 min, respectively. Thus, it was concluded that glycerol penetrates the thylakoid membranes within seconds, while sucrose and glucose act osmotically, inducing water efflux, with trans-membrane diffusion half-times of the saccharides in the time range of tens of minutes to hours.

Since cytochrome *f* oxidation depends on the diffusion of oxidized plastocyanin in the lumenal compartment, the proportional relationship between the rate of cytochrome *f* oxidation and ambient viscosity indicates that with glycerol as viscosogenic agent, the lumenal and external viscosities were identical due to fast influx of glycerol into the lumenal compartment. The tendency of glycerol to replace water molecules on the protein surface (*33*) would thus account for the sluggish reversibility of the inhibition by glycerol mentioned above.

For sucrose and glucose, transmembrane transport is extremely slow, judging from the scattering experiments described above. Therefore, the inhibition of cytochrome *f* oxidation by the saccharides probably was caused by an osmotically induced water efflux, with the consequence that the natural lumenal viscosogenic agents (sugars and amino acids) were concentrated, thus increasing lumenal viscosity. This assumption explains the observation that the concentration dependence of the inhibition of cytochrome *f* oxidation was similar for sucrose and glucose (Figure 2), while their viscosity dependence differed from each other (Figure 3B) due to their different relationships between concentration and viscosity (*27*).

Since the relative amount and composition of saccharides in chloroplasts, and, therefore, in the lumenal compartment, is known to vary greatly with seasonal and diurnal rhythm (*34*), the results with sucrose and glucose shown in Figures 2 and 3B cannot be generalized. Rather, the relationship between lumenal viscosity and external concentration has to be established for each case. In this context, it is also noteworthy that at lower temperatures, glycerol sometimes seemed to osmotically influence lumenal viscosity. At 10 and 15 °C, half-times as large as 25 ms have been observed

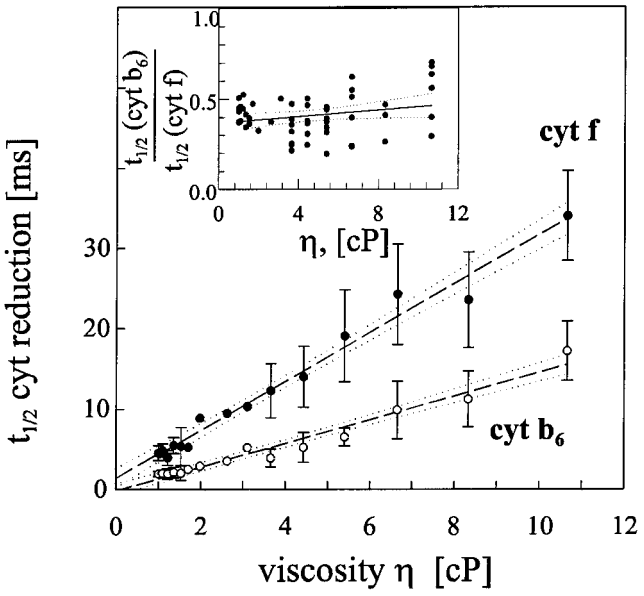

FIGURE 5: Half-time of reduction of cytochromes b_6 (open symbols) and *f* (closed symbols), and their ratio (inset) as a function of ambient viscosity. All experiments conducted with glycerol as viscosogenic substance; conditions as in Figure 1. Linear $t_{1/2}/\eta$ regression fit for cytochrome b_6, $t_{1/2} = 1.5\eta - 0.2$; for cytochrome *f*, $t_{1/2} = 3.0\eta + 1.3$. Confidence interval is 95%.

for flash-induced cytochrome *f oxidation* at concentrations below 4 M glycerol (data not shown). However, most results obtained in the presence of glycerol were in accordance with the assumption that internal and external viscosities were identical.

Viscosogenic Inhibition of ISP Movement. As demonstrated in Figure 1, not only cytochrome *f* oxidation but also the rates of reduction of cytochromes b_6 and *f* are sensitive to viscosity. Using glycerol as viscosogenic agent, a linear relationship was observed between viscosity and the reduction half-times of both cytochromes (Figure 5). As can be seen in the inset of Figure 5 where the ratio of the reduction half-times of cytochromes b_6 and *f* is shown, reduction of cytochrome b_6 was faster than that of cytochrome *f* reduction in every experiment. In fact, reduction of cytochrome b_6 was, on the average, approximately twice as fast as that of cytochrome *f*, regardless of ambient viscosity.

It is important to ensure that the effects of ambient viscosity on the kinetics of the redox events were not a consequence of a global effect on the thylakoid membrane. It has been noted above that, while the slow phase of P_{700}^+ reduction, which requires binding of plastocyanin, was sensitive to a viscosity increase through added glycerol, the 12 μs fast phase (reduction by bound plastocyanin) was not (*20*), demonstrating the selective inhibitory effect of high viscosity on electron transport events in situ.

Viscosity-Independent Redox Reactions of the Cyt b_6f Complex. To further ensure there is no global viscosogenic effect specifically on the cyt b_6f complex, we sought to find a redox reaction that does not involve movement of the reactant protein(s) through the aqueous phase, and whose kinetics are consequently viscosity-independent. The reduction kinetics of cytochrome b_6 under oxidizing conditions were found to be constant over a viscosity range from $\eta =$ 1 to 3.6 cP (Figure 6). Oxidizing conditions normally induce "state-s" (*35*), in which the rate of cytochrome b_6 oxidation

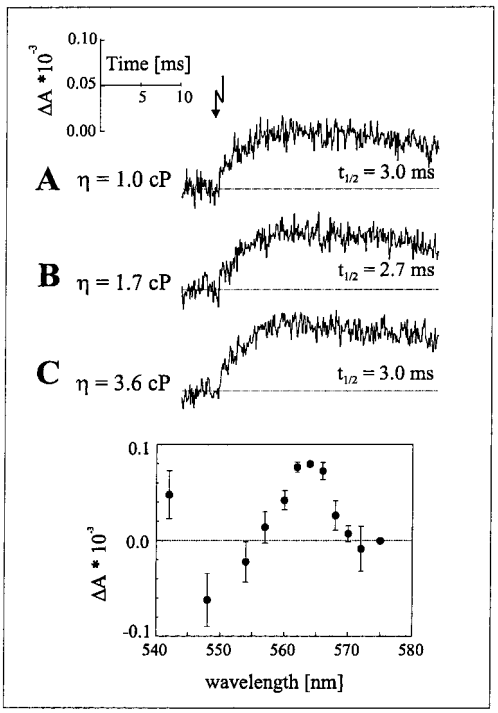

FIGURE 6: Kinetics of flash-induced cytochrome b_6 reduction under oxidizing conditions. (A) Control, no glycerol added, $\eta = 1$ cP. (B, C) In the presence of 20% (w/w) glycerol, $\eta = 1.7$ cP, and 40% (w/w) glycerol, $\eta = 3.6$ cP, respectively. Each curve is the average of 500 measurements on 10 samples. Inset: Difference spectrum of cytochrome b_6 reduction at oxidizing conditions in the absence of glycerol, $\eta = 1$ cP. The spectrum is normalized to 564 nm, with 575 nm as the reference wavelength. Each data point is the average of 216 measurements on 6 samples. Conditions as described in Figure 1, except that duroquinol was omitted, and continuous far-red illumination (60 μE m^{-2} s^{-1}) was applied.

is inhibited by an unknown mechanism. However, development of "state-s" can be minimized by application of continuous far-red light while simultaneously providing oxidizing conditions. Under these conditions, the amplitude of cytochrome b_6 reduction was much smaller (ca. 15%) than measured under reducing conditions. The difference spectrum (inset of Figure 6) demonstrates that these absorbance changes are mainly derived from reduction of the cytochrome. A half-time of 2.7−3.0 ms was determined for cytochrome b_6 reduction, with no discernible effect of ambient viscosity (Figure 6A−C), demonstrating that flash-induced cytochrome b_6 reduction is not sensitive to increased lumenal viscosity when the high potential site (ISP, cytochrome *f*) is oxidized.

It is important to note that measurements of the kinetics of reduction of cytochrome b_6 as well as cytochrome *f* were made in the presence of the inhibitor NQNO (0.35 μM NQNO), whose major effect has been considered to be the increase of the amplitude of cytochrome b_6 reduction (*22*, *24*). This is thought to result from a primary site of action in the quinone binding (Q_N) niche in the b_6f complex that inhibits reoxidation of cytochrome b_6. It was also reported that the reduction rate of cytochrome *f* was highly sensitive to NQNO, but that the initial rate of reduction of cytochrome b_6 was affected only at concentrations of >10 μM NQNO (*25*). We have found, however, that the inhibition of reduction rates of cytochromes b_6 and *f* by NQNO is similar (K_I ca. 0.35 μM) and proportional to the increase in amplitude of cytochrome b_6 reduction (data not shown). According to the Q-cycle model with a single site of inhibition by NQNO at the Q_N site in the low potential chain, one would expect that the subsequent cytochrome *f* reduction would be inhibited, or delayed, only by the time interval over which cytochrome b_6 reduction is retarded. Triphenyltin-chloride (TPT) and Valinomycin/K^+, also putative Q_N inhibitors (*26*), had the same quantitative effect on cytochrome *f* reduction; the observed retardation of cytochrome *f* reduction was proportional to the amplitude of cytochrome b_6 reduction. Therefore, the proportional inhibition of cytochromes b_6 and *f* reduction by Q_N inhibitors (data not shown) seems to be a genuine Q_N effect, but cannot be explained by the bound inhibitor simply blocking cytochrome b_6 reoxidation. It also requires the assumption that Q_N inhibitors have an additional inhibitory effect of a global nature that influences the binding of the ISP to both its binding sites (manuscript in preparation).

DISCUSSION

Viscosogenic Effects on Diffusion of Soluble Macromolecules. According to the Stokes−Einstein relation, the molecular diffusion coefficient (*D*) is inversely proportional to the ambient viscosity, η:

$$D = \frac{kT}{(f/f_0)6\pi\eta a} \quad (1)$$

with *a* the average radius of the molecule and f/f_0 a geometrical correction factor for nonspherical molecules. In diffusion-limited reactions, macromolecules are assumed to adhere to Kramers' law (*36*) that their rate of reaction, *k*, is inversely proportional to the viscosity, η, and consequently the half-time, $t_{1/2}$, is proportional to η:

$$k \propto \eta^{-1} \Leftrightarrow t_{1/2} \propto \eta \quad (2)$$

The half-time of flash-induced cytochrome *f* oxidation, which involves long-range ($\sim 10^2-10^3$ Å) diffusion of oxidized plastocyanin from PS I to the cytochrome b_6f complex, is proportional to viscosity (Figure 3A) and is consistent with the observation that this reaction is diffusion-limited (*22*). The same linear viscosity dependence has been found for reduction of P_{700}^+ by added plastocyanin in PS I enriched subchloroplast particles (*37*).

Motion of ISP. The concept that the ISP shuttles back and forth between cytochrome *f* and the Q_P site was derived from structure analysis of the bc_1 complex (*14*−*16*). Additional evidence supporting the mechanism was based on the effect of mutational alteration of the presumed pivot region of the ISP in the bacterial bc_1 complex (*8*). It can therefore be assumed that the viscosogenic inhibition observed for reduction of cytochrome b_6 as well as cytochrome *f* can be traced to the movement of the soluble domain between two sites as proposed in (*14*−*16*). For the chloroplast b_6f complex, it is known that the soluble domain of the ISP that contains the iron−sulfur cluster contains at least 139 of the 179 amino acids of the mature protein (*38*). Analysis of the EPR signal associated with the [2Fe-2S] cluster of ISP in oriented membranes indicates that there are also two different binding sites for ISP that result in a different orientation of its *g* tensor (*12*). From the viscosity dependence of ISP-dependent

reactions, it is inferred in the present study that ISP movement is necessary for electron transfer in the b_6f complex. Furthermore, the viscosity dependence of the ISP motion is very similar to that of plastocyanin, which is known to mediate the diffusion-limited oxidation of cytochrome *f* (*22*).

It has been experimentally found that the rate constants, *k*, of intramolecular movements follows

$$k \propto \eta^{-\gamma} \Leftrightarrow t_{1/2} \propto \eta^{\gamma} \qquad (3)$$

with γ (normally but not always ≤ 1) depending on the nature of the movement (*39, 40*). Conformational changes which have little influence on the surface tend to be independent of viscosity ($\gamma \rightarrow 0$), while conformational changes that involve major surface changes, as, e.g., the folding rate of DNA coils (*41*), tend to show a pronounced viscosity dependence ($\gamma \rightarrow 1$) (*39*).

The observed relationship of 1:2 for reduction half-times of cytochromes b_6 and *f*, which depend on the movement of the soluble domain of ISP, are also linearly proportional to viscosity ($\gamma = 1$). The soluble domain of ISP is tethered to the b_6f complex by a polypeptide chain with significant conformational freedom (*38, 42*). Thus, the soluble domain of ISP is only loosely connected to the integral part of the b_6f complex, and despite the confinement to the binding niche, its movement resembles that of soluble proteins, with the viscosogenic inhibition therefore also resembling that of soluble proteins.

Osmotic Effects. Glycerol usually is not osmotically active, but readily penetrates the thylakoid membrane so that internal viscosity and external viscosity are identical (Figure 4). With saccharides, however, an osmotically induced water transport across the thylakoid membrane is responsible for most or all of the increased lumenal viscosity. Under these conditions, internal viscosity and external viscosity deviate from each other. Since plastocyanin is a lumenal protein, the lumenal, not the external, viscosity is relevant for its effect on the rates of cytochrome *f* oxidation. This explains the observation that with sucrose and glucose, inhibition of cytochrome *f* oxidation is not proportional to the external viscosity (Figure 3B).

Consequences for the Concerted Reduction of the High and Low Potential Chains by Plastoquinol. Although concerted electron transfer from the quinol at the Q_P site to cytochromes b_6 and *f* has been assumed to be an integral aspect of the mechanism of oxidant-induced reduction and the Q-cycle (*43*), consideration of the multiple steps involved in the transfer of electron from the quinol to cytochrome *f*, and possibly to cytochrome b_6 as well, makes such a concerted model less of a conceptual imperative even with the assumption of an obligate Q-cycle mechanism. A faster reduction of cytochrome b_6 compared to cytochrome *f* was first reported in spinach chloroplasts (*24*). A larger difference in the rates of reduction was found in mutants of cytochrome *f* in *Chlamydomonas reinhardtii* that affected its N-terminus (*44*) or conserved residues that H-bond to the internal H_2O chain (*45*).

Discrete Positions of the Oxidized and Reduced ISP. (A) Oxidizing Conditions. The reduction of cytochrome b_6 is not sensitive to ambient viscosity (Figure 6). Because reduction of cytochrome b_6 in the low potential chain cannot precede

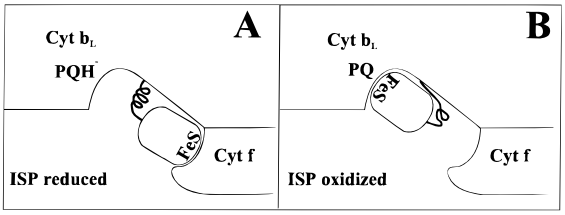

FIGURE 7: Model for movement of ISP between Q_P and cytochrome *f*, based on monoexponential reduction of up to 100% cyt b_6, and 2:1 ratio of rates of cyt b_6:*f* reduction. (A) Reduced ISP is bound to cytochrome *f*, and (B) oxidized ISP is bound to the Q_P binding site, the two extreme positions in the movement of the ISP.

that of ISP in the high potential chain for thermodynamic reasons, it is concluded that the oxidized ISP is already bound to the Q_P site (Figure 7B).

(B) Reducing Conditions. The high potential chain is partially oxidized by plastocyanin, followed by the reduction of ISP and cytochrome b_6 at the Q_P site by plastoquinol. The viscosogenic inhibition of cytochrome b_6 reduction indicates that prior to the flash, the ISP was not bound at the Q_P niche. Since most experiments were conducted in the presence of duroquinol, it can be assumed that the ISP was reduced prior to flash illumination. The requirement of a time interval for cytochrome *f* reduction approximately twice as long as that for cytochrome b_6 implies that completion of the reduction pathway requires completion (Figure 7A) of the second half of the motional cycle.

Amplitude of Cytochrome f Oxidation. With thylakoids, flash-induced oxidation of cytochrome *f* accounts for only ca. 50−60% of its chemical content (e.g., see refs *21, 26*). The amplitude of monoexponential reduction of cytochrome b_6 is larger than that of cytochrome *f* in the presence of Q_N inhibitors (e.g., Figure 1A; see also ref *26*), indicating that the number of positive charges in the low potential chain is larger than inferred from the amplitude of cytochrome *f* oxidation. Assuming a serial oxidation of cytochrome *f* and ISP by plastocyanin (i.e., obligatory electron flow from ISP → cytochrome *f* → plastocyanin, without parallel oxidation of cytochrome *f* and ISP), the small amplitude of observable cytochrome *f* oxidation suggests (i) that reduced ISP is bound to cytochrome *f* and can rapidly (<100 μs) rereduce the heme (*46, 47*). The observed 2−3 ms half-time of cytochrome *f* reduction is attributed to a second oxidation (double hit) that occurs after ISP oxidation. It is inferred that the 2−3 ms is the time required for the ISP round-trip transit from cyt *f* to Q_P and back. The amplitude of the millisecond phase would be determined by PS I:b_6f stoichiometry, the specific lateral distribution of PS I and b_6f complex, and the flash intensity. Alternatively, (ii) parallel oxidation of cytochrome *f* and ISP by plastocyanin might also explain incomplete oxidation of cytochrome *f* (*21*); in this case, the ISP does not have to be bound to cytochrome *f* for rapid oxidation. Presently, we cannot distinguish between (i) and (ii): On one hand, the double-hit explanation seems to be in conflict with the results from experiments at low flash intensity where the amplitudes of cytochrome *f* oxidation and cytochrome b_6 reduction decreased approximately by the same factor (data not shown); on the other hand, a pathway for parallel oxidation of ISP and cytochrome *f* is not consistent with the 2:1 ratio in cytochrome b_6:*f* reduction rates, but is consistent with a

pathway of ISP movement that involves translation just between its Q_P and cytochrome *f* proximal binding sites.

Consequences of ISP Motion: The Rate-Limiting Step. From studies of the bc_1 complex, isolated and in membranes, from mitochondria and photosynthetic bacteria, three different steps in the oxidation of the bound ubiquinol have been proposed to provide the rate-limiting step of the process: (i) deprotonation of the neutral quinol to the anionic quinol (*4*, *5*); (ii) electron transfer from the quinol to the ISP (*10*, *11*); and (iii) tethered diffusion of the ISP between sites proximal to Q_P and cytochrome c_1 (*8*). Step (i) was inferred from (a) the thermodynamic requirement that the neutral quinol be deprotonated in order to allow generation of a reductant strong enough to reduce the ISP (*48*), and (b) the pH dependence of the activation energy for net electron transfer to the complex, indicative of the release of one proton per quinol (*4*). Step (ii) was proposed as a result of the findings (a) that the rate of net electron transfer through the complex was affected by the midpoint potential of the ISP in a manner qualitatively consistent with control by the electron-transfer rate from quinol to ISP (*49*, *50*), and (b) that the activation energy for the net electron-transport rate through the complex in *Rb. sphaeroides* was not pH-dependent (*10*). (iii) A role of constrained diffusion of ISP in the limiting process could be inferred from the finding that the activation energy for net electron transfer was increased approximately 3-fold in *Rb. sphaeroides* by mutations that should limit the flexibility of the ISP soluble domain.

The present studies imply that constrained diffusion of the ISP, mechanism (iii) above, is part of the rate-limiting step in the turnover of the cyt b_6f complex. In our experiments, we can estimate the characteristic time for the viscosity-independent step in quinol oxidation, which is obtained by extrapolating the regression line for cytochrome b_6 reduction to zero viscosity. The value of the intercept is <0.5 ms, and possibly much lower, whereas the viscosity-dependent ISP movement (including binding) has a slope of 1.5 ms·cP^{-1} for cytochrome b_6 reduction, and 3.0 ms·cP^{-1} for cytochrome *f* reduction (Figure 5). Due to this 1:2 ratio, this viscosity-dependent process is most readily associated with the constrained motion of the ISP, including the binding process.

However, we do not wish to exclude at this stage the possibility that steps (i) and/or (ii) have half-times of the same order of magnitude and can also participate in the rate limitation. In particular, we have found that the activation energy for electron transfer through the b_6f complex is significantly pH dependent over the physiological range (G. M. Soriano and W. A. Cramer, unpublished data). Thus, we suggest, not only for diplomatic reasons, that steps (i)−(iii) described above may all have rates near the millisecond time range, and, depending on the exact experimental conditions, may exert partial control of the rate of charge-transfer processes through the bc_1 and b_6f complexes.

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