On the Question of Interheme Electron Transfer in the Chloroplast Cytochrome b_6 in Situ†

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ABSTRACT: The redox properties, the site of action of the inhibitor NQNO, and the question of interheme transfer in the chloroplast cytochrome b_6 have been examined with regard to the role of the b_6–f complex in quinol oxidation and H^+ translocation. (i) The two hemes of the cytochrome, b_n and b_p, have similar ($\Delta E_m \leq 50$ mV) oxidation–reduction midpoint potentials that are pH-independent in the range pH 6.5–8.0 ($E_{m7} = -40$ mV) but are pH dependent below this range with an estimated p$K = 6.7$. (ii) Only half of cytochrome b_6, the stromal-side heme, b_n, was reducible by NADPH and ferredoxin. (iii) The 2–3-fold increase (to 0.60 ± 0.09 heme/600 Chl) in the amplitude of flash-induced cytochrome reduction caused by NQNO was not affected when heme b_n was initially reduced, implying that NQNO affects flash reduction at the site of heme b_p. (iv) Multiple light flashes did not increase the amplitude of b_6 reduction in the presence or absence of NQNO or show binary oscillations. Together with localization of a site of action of NQNO near heme b_p, these data provide no evidence for efficient electron transfer from heme b_p to heme b_n as specified by the Q cycle model. (v) NQNO interaction with heme b_p does not block its oxidation, since reoxidation of the flash-reduced cytochrome in its presence or absence was 4–5 times faster ($t_{1/2} \approx 30$ ms) when heme b_n was reduced. The faster oxidation of the photoreduced cytochrome after NADPH–Fd reduction of heme b_n indicates that the oxidation of b_n and b_p may be cooperative.

Comparative Biochemistry and Structure of Cytochrome b_6–f and b–c_1 Complexes. The ubiquinol:plastoquinol:plastocyanin (b_6–f) oxidoreductase or cytochrome c oxidoreductase (b–c_1) is present in a broad spectrum of energy-transducing organisms and organelles. These complexes provide the pathway for transferring electrons from ubiquinol or plastoquinol to the distributive carrier cytochrome c or plastocyanin (Hauska et al., 1983; Rich, 1984; Crofts, 1985; Dutton, 1986; Cramer et al., 1987; O'Keefe, 1988). At the same time they act to translocate protons across the membrane. For the mitochondrial b–c_1 complex there is general agreement that two protons are translocated for each electron transported through the complex, i.e., $H^+/e^- = 2$ (Wikström & Saraste, 1984). Although similar $H^+/e^- = 2$ ratios have been observed in chloroplasts in the presence of low values of the $\Delta\tilde{\mu}_{H^+}$, it is not clear that an $H^+/e^- = 2$ ratio persists in the presence of steady-state values of ΔpH or $\Delta\tilde{\mu}_{H^+}$ [cf. Graan and Ort (1983), Table IX of Cramer et al. (1987), Hangarter et al. (1987), and Rich (1988)].

The amino acid sequence and distribution of hydrophobic residues of the heme-binding domain of the cytochrome b polypeptide were shown to have a high degree of identity between mitochondria across the phyla and between chloroplasts and photosynthetic bacteria (Widger et al., 1984; Saraste, 1984; Davidson & Daldal, 1987; Hauska et al., 1988). However, cytochrome b_6 is approximately half the size of the analogous mitochondrial and chromatophore b cytochromes, its 214 residues encompassing only the heme-binding domain. Subunit IV of the chloroplast b_6–f complex may contain one or more of the functions in the COOH-terminal half of the larger b cytochromes (Widger et al., 1984), including the quinone binding function (Doyle et al., 1989). The loss of antimycin A sensitivity may be a consequence of the truncated nature of the b_6 polypeptide as well as specific residue changes near the NH_2-terminus (Howell & Gilbert, 1988). In addition, His residues 186 and 201 that coordinate the two hemes are separated by 14 residues in cytochrome b_6, compared to 13 in the b–c_1 complexes, which is perhaps responsible for the smaller difference in E_m as well as visible and EPR spectral peak values of the chloroplast cytochrome (Widger et al., 1984). It was inferred that two hemes span the membrane, one on each side of the bilayer, cross-linking two *trans*membrane hydrophobic α-helical peptides. This structure provided a framework for a *trans*membrane electron transport pathway (Widger et al., 1984; Saraste, 1984).

Q Cycle Pathway of Electron Transport. A Q cycle model involving interheme electron transfer between two cytochrome b, or two hemes of a cytochrome b, was proposed (Mitchell, 1976) to explain the observation of oxidant-induced reduction of the mitochondrial cytochrome b (Wikström & Berden, 1972) and to accommodate its dependence on the presence of oxidized iron–sulfur protein in the b–c_1 (Trumpower, 1981) or b_6–f complex (Prince et al., 1982) and the $H^+/e = 2$ ratio in mitochondria associated with electrogenic H^+ translocation. The pathway in the b–c_1 complex is characterized by a one-electron oxidation of quinol to semiquinone by the [2Fe-2S] acceptor, semiquinone reduction of a low-potential heme, interheme transfer (West et al., 1988) across ~12 Å to the high-potential heme, and then an antimycin A sensitive oxidation of the latter heme that transfers two electrons in succession to reduce ubiquinone to ubiquinol. Part of the driving potential for the cycle would arise from the positive potential difference, $\Delta E_m \approx 0.15$ V, between these hemes in mammalian mitochondria and chromatophores (Crofts, 1985).

The existence of a Q cycle in chromatophores and chloroplasts was inferred from the observation of a slow (millisecond) electrochromic carotenoid band shift (Jackson & Dutton, 1973;

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Joliot & Delosme, 1974; Glaser & Crofts, 1984; Jones & Whitmarsh, 1985; Robertson & Dutton, 1988) that occurs on approximately the same time scale as the reduction of cytochrome *b*. The occurrence of interheme electron transfer in chromatophores of *Rhodobacter sphaeroides* was shown by separation of the flash-induced reduction of the hemes *b*-561 and *b*-566, in the presence of antimycin A, by flash number and ambient redox potential (Meinhardt & Crofts, 1983). Quinone analogues that act in mitochondria and chromatophores as inhibitors on each side of the membrane near b_p and b_n[1] [summarized in Cramer et al. (1987)] and the detection of a bound semiquinone on the n side of the membrane (Ohnishi & Trumpower, 1980; Robertson et al., 1984) are consistent with a Q cycle. Use of the inhibitor antimycin A that acts at the quinone binding site near heme b_n and blocks its oxidation has been a crucial inhibitor in studies of the cytochrome *b* pathway in mitochondria and chromatophores, allowing observation of the light-induced cytochrome *b* reduction which is of small amplitude in the absence of antimycin (Matsuura et al., 1983; Meinhardt & Crofts, 1983).

Question of a Q Cycle in Chloroplasts. The similarity of the room temperature α-band maxima [$\Delta\lambda_m \approx 1$ nm; Nitschke et al., 1988; Joliot & Joliot, 1986, 1988] of the two hemes makes it difficult to determine which heme is reduced in a light flash and whether interheme transfer occurs, and this difficulty is compounded by the similarity of the in situ E_m values documented below. A Q cycle mechanism in chloroplasts is suggested by analogy with chromatophores and mitochondria and by (i) kinetic correlation of the slow electrochromic phase (Joliot & Delosme, 1974) with turnover of the b_6–f complex (Velthuys, 1979; Joliot & Joliot, 1986), (ii) a pre-steady-state H^+/e ratio = 1.7–1.9 in situ for the b_6–f complex (Hangarter et al., 1987), with transfer of the extra H^+ obligatorily coupled to electron transfer (Rich, 1988) or coupled under conditions of low $\Delta\tilde{\mu}_{H^+}$ [e.g., Bouges-Bocquet (1981) and Hope and Matthews (1987)], (iii) reconstitution of electrogenic H^+ translocation with $H^+/e \approx 2$ (Hurt et al., 1982; Willms et al., 1988), and (iv) the increase in amplitude of cytochrome b_6 photoreduction and inhibition of the slow electrochromic phase by the compound NQNO, which has been proposed to act as an analogue to antimycin A (Selak & Whitmarsh, 1982). The observed effect of antimycin A itself on cytochrome b_6–f is either nil or different from that observed in mitochondria and bacteria (de Wolf et al., 1988).

Points i–iii do not uniquely define a Q cycle mechanism and are also consistent with a proton pump mechanism of H^+ translocation for which there is a precedent in cytochrome oxidase (Wikström & Saraste, 1984) and bacteriorhodopsin (Khorana, 1988). A lack of effect of extraction of endogenous quinone on electron transfer between cytochrome *b* and the iron–sulfur center was found to be inconsistent with details of the Q cycle mechanism in the yeast mitochondrial b–c_1 complex (Tsai et al., 1987). The conclusion (point iv) that the action of NQNO on the chloroplast b_6–f complex is analogous to that of antimycin A in mitochondria and chromatophores is examined in the present work, along with the question of interheme electron transfer.

Materials and Methods

Chloroplast Preparation. Chloroplasts were prepared from 1–3-month-old spinach seedlings (variety Longstanding Bloomsdale Dark Green) grown at 18–25 °C with 12 h per day of fluorescent and incandescent light in a growth chamber. The preparation and resuspension of chloroplasts were described in Girvin and Cramer (1984), except that the resuspension included 5 mM instead of 2 mM $MgCl_2$. All reactions were assayed in a medium containing 0.2 M sucrose, 30 mM Tricine–KOH, pH 7.8, 5 mM $MgCl_2$, and 25 mM KCl. Anaerobic conditions resulted from addition of 160 units of glucose oxidase (Sigma G-8135) and 5.5 mM glucose. NADPH was purchased from Sigma (N-1630). Ferredoxin and NQNO were generous gifts of M. Black and J. Whitmarsh, respectively.

Redox Titrations. The following mediators (midpoint potentials; Clark, 1960; Böhme & Cramer, 1973) were used (20 μM, except where noted); 1,2-naphthoquinone (E_{m7} = +135 mV), 40 μM 1,4-naphthoquinone (E_{m7} = +65 mV), menadione (E_{m7} = 0 mV), 2,5-dihydroxy-1,4-benzoquione (E_{m7} = −60 mV), anthraquinone-2,6-disulfonate (E_{m7} = −185 mV), and anthraquinone-2-sulfonate (E_{m7} = −225 mV). DCMU (3 μM), methylviologen (0.1 mM), and gramicidin (5 μM) were present in all samples. Ambient redox potentials were measured with a Metrohm AG9100 PT:Ag|AgCl redox electrode and a Corning Digital III millivolt meter. The potentials were adjusted by injection of small volumes of sodium dithionite or potassium ferricyanide as reductant or oxidant. Anaerobic conditions were maintained by flushing the cuvette with water-saturated argon (Chemetron Corp., Chicago, IL). The system was calibrated at high potentials with saturated quinhydrone (Clark, 1960) and its accuracy checked at negative potentials by titration of a 20 μM FMN solution (Cramer & Butler, 1969), which had a midpoint of −205 mV and a slope corresponding to $n = 2$ in the Nernst equation. Titrations were performed in a modified dual-wavelength spectrophotometer with a measuring beam intensity of 10–20 nE/m^2-s at 560 nm (Girvin & Cramer, 1984).

Chemical Difference Spectra. Measurements were made on an Aminco dual-wavelength monochromator with a computer- (IBM AT) controlled stepper motor (High-Sync AC synchronous/DC stepper motor; Bodine Electric Co.). The amplifier time constant was 0.33 ms. The measuring beam light, after passing through a vertical cuvette (Butler, 1972), was blocked by a Balzer's DT-Gruen filter to prevent any interference from fluorescence. Difference spectra were obtained by subtracting the spectrum of the initial state from that arising from further additions. Samples were mixed before scanning with a magnetic stirrer that was turned off before measurement of a spectrum. In order to reduce drift in the signal during each scan due to sample settling, each sample was allowed to settle 1 min before scanning after the stirrer was turned off.

Cytochrome Photoreduction. Measurements were made with an SLM (Urbana, IL) monochromator using a half-bandwidth of 2 nm, with the measuring lamp output shuttered to avoid possible actinic effects, and EMI 9524B photomultiplier as detector coupled to a homemade amplifier and offset module, and a Nicolet 11/70 signal averager. The shutter opened 250 ms before the measurement sweep of the Nicolet 11/70 signal averager began and closed within 50 ms after the end of the sweep. Balzer's DT-Gruen and Corning 4-96 filters were placed between the sample and photomultiplier

[1] Abbreviations: b_n and b_p, the two hemes of cytochrome b_6 positioned respectively on the sides of the membrane with negative and positive values of the proton electrochemical potential, $\tilde{\mu}_{H^+}$; (H)NQNO, 2-*n*-(heptyl)nonyl-4-hydroxyquinoline *N*-oxide; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; Fd, ferredoxin; λ_m, wavelength of absorbance maximum; ϵ_{mM}, millimolar extinction coefficient; 1,2-NQ, 1,2-naphthoquinone; DQH_2, duroquinol; DNP-INT, 2-iodo-6-isopropyl-3-methyl-4-nitrophenyl 2,4-dinitrophenyl ether; PQ, PQH_2, and $PQ^{\cdot-}$, plastoquinone, plastoquinol, and anionic plastosemiquinone radical; PS I, photosystem I.

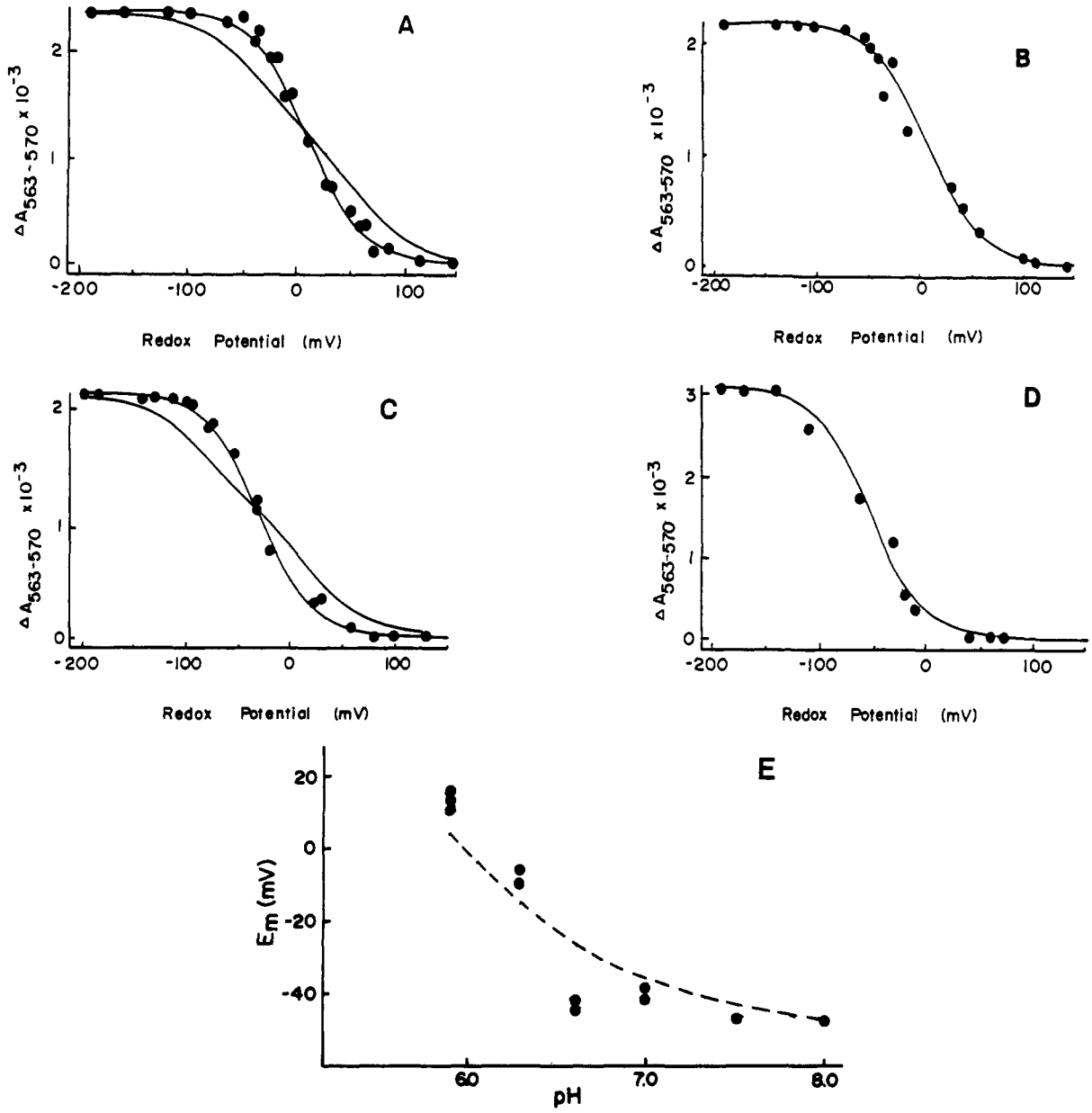

FIGURE 1: Redox titrations of cytochrome b_6 at pH 5.9 (A), 6.3 (B), 7.0 (C), and 8.0 (D) to which $n = 1$ Nernst plots were fit by assuming E_m values of +13, −10, −40, and −45 mV, respectively. The titrations were carried out in the reductive direction with the wavelength pair 563 and 570 nm. Also shown in (A) and (C) are titration curves (solid curves without data points) for a two-component system with midpoint $E_{m1} = E_m + 50$ mV and $E_{m2} = E_m - 50$ mV, where E_m is the midpoint of the $n = 1$ titration. Chl concentration [μg/mL]: 63 (A), 61 (B), 50 (C), and 67 (D). Absorbance change corresponds to 2.0 hemes/600 Chl. (E) pH dependence of the midpoint potential of $n = 1$ Nernst plots for cytochrome b_6, including titrations at pH 6.6 and 7.5 (not shown in panels A–D); data are fit (dashed line) also by assuming that the pK of the reduced form = 6.7 and the limiting E_m at high pH = −47 mV.

tube to block possible fluorescence. The laser (Phase-R DL-1100, lasing dye sulfrhodamine 640) (Figures 3 and 5) and xenon (EG & G FX193 with FY703 Lite-Pak) flash (Figure 6) outputs were filtered (Corning 2-59) and adjusted to yield a saturating or threshold saturating response, respectively. Repetition of the xenon flash train (single, double, or multiple) was at a frequency of 0.1 Hz, with an amplifier time constant of 0.3 ms. The amount of heme reduced by flash, or in the dark by NADPH or dithionite, was calculated from a difference molar extinction coefficient of 14[$\Delta\epsilon_{mM}$(563–570)] applied to the data of Figure 1 and 17[$\Delta\epsilon_{mM}$(563–572)] used elsewhere. These values were derived by Cramer and Whitmarsh (1977) from the data of Stuart and Wasserman (1973) or by analogy from data on the chloroplast cytochrome *b*-559 (Cramer et al., 1986). At the present time, extinction coefficient data from a purified cytochrome b_6 preparation are not available.

Results

Redox Titration of Cytochrome b_6. The E_m values in situ of the two hemes of cytochrome b_6 were titrated in the presence of gramicidin at six different pH values in the dark (Figure 1A–D, for pH values 5.9, 6.3, 7.0, and 8.0). In all cases, the data were fit by an $n = 1$ Nernst plot with single E_m values of +13, −10, −40, and −45 mV, respectively, for the titrations shown in figure 1A–D. The curves without data points shown in Figure 1A,C represent simulated titrations assuming two components with midpoints = $E_m \pm 50$ mV whose midpoints differed by 100 mV. It is concluded that the E_m values of cytochrome b_6 are isopotential within 50 mV. In contrast, two distinct E_m values, separated by $\Delta E_m = 0.10$–0.14 V, have been measured with the isolated cytochrome b_6–f complex in vitro [summarized in Cramer et al. (1987)]. The E_m values obtained in the present work are approximately independent of

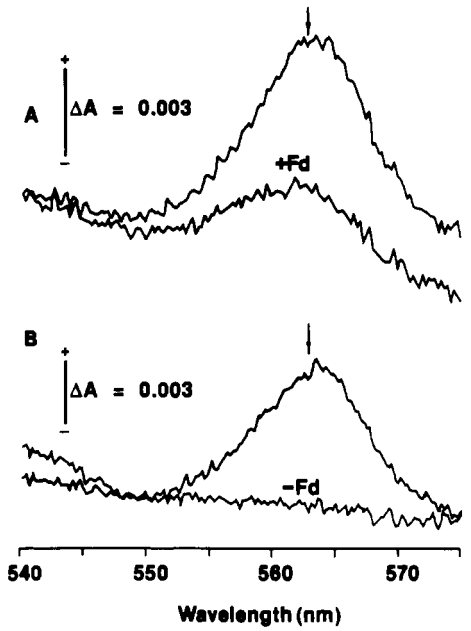

FIGURE 2: Comparison of chemical (dithionite) and enzymatic (NADPH–ferredoxin) reduction of cytochrome b_6. Chloroplasts were resuspended at a concentration of 80 μg/mL in a vertical curvette, with an optical length of 1.4 cm, in the presence of 1 μM DBMIB and 0.7 mM NADPH. To obtain semianaerobic conditions, 160 units of glucose oxidase and 5 mM glucose were added. Ascorbate (5 mM) was also present to minimize or eliminate any contribution of cytochromes f or b-559 to the spectra. In (A), 5 μM ferredoxin was also added. In (A) and (B), the upper trace shows the addition of dithionite and 10 μM anthraquinone to fully reduce both hemes of cytochrome b_6. The lower traces in (A) and (B) result from addition of NADPH in the presence (A) and absence (B) of ferredoxin. Traces are an average of 200 sampling events at each wavelength, with an amplifier time constant of 0.33 ms.

pH above pH 6.5–7.0, but three independent titrations at pH 5.9 and two at pH 6.3 indicate a pH dependence of approximately –60 mV per pH unit below pH 6.6. The dashed curve in Figure 1E shows a fit to the data assuming a pK = 6.7 for the reduced form. The determination of the pH dependence required measurements in a narrow pH window, below pH 6.5 but not too close to acidic pH values at which thylakoids slowly precipitate. The E_m in lettuce chloroplasts was reported to be pH independent over an unspecified range but to be approximately 50 mV more positive at pH 6 than at pH 7.5–8 in lettuce b_6–f complex (Rich & Bendall, 1980) and to be pH dependent in the spinach complex (Hurt & Hauska, 1983).

Reduction of Half of Cytochrome b_6 by NADPH–Ferredoxin. Dithionite in the presence of anthraquinone as a lipophilic redox mediator caused the reduction of 1.95 ± 0.20 (four trials) hemes/600 Chl (Figure 2A). The reduction in the absence of the mediator is incomplete (Widger et al., 1983), indicating that at least one heme has a limited accessibility to dithionite. The amplitude of the cytochrome b_6 heme reduction by NADPH in the presence of ferredoxin is approximately 1.08 ± 0.09 (three trials) heme equiv/600 Chl, half of that obtained in the presence of dithionite (Figure 2A). Reduction of cytochrome b_6 by NADPH requires ferredoxin (Figure 2B), as noted previously by others using chloroplasts (Arnon & Chain, 1979; Telfer & Barber, 1981) or isolated b_6–f complex (Lam & Malkin, 1982; O'Keefe, 1983) for which the presence of the inhibitor DNP-INT was required.

NADPH–Ferredoxin Reduces Heme b_n. The pathway of cytochrome b_6 reduction by NADPH–ferredoxin is through the outside heme, since the reaction is not affected by the presence of the inhibitor DBMIB for the b_6–f complex that was present for the experiments shown in Figure 2. Therefore, the 1 heme equiv or 50% of the cytochrome b_6 that is reduced must be either (i) the stromal-side heme b_n in all complexes or (ii) a 50:50 mixture of fully oxidized and fully reduced complexes, the latter perhaps because of thylakoid membrane heterogeneity. These two possibilities can be tested by measuring the amplitude of the reduction by a single flash after prereduction by NADPH–Fd. This amplitude would be smaller by a factor of 2 if half of the complexes are fully reduced but unaffected if b_n is initially reduced and b_p is oxidized in all complexes.

The amplitude of the flash-induced reduction of cytochrome b_6 is negligible unless the quinone pool is reduced (Velthuys, 1979), which can be accomplished by addition of duroquinol (DQH_2) (Selak & Whitmarsh, 1982). Addition of NQNO results in a further increase of a factor of 2–3 in the amplitude (Selak & Whitmarsh, 1982). The amplitude of cytochrome b_6 reduction by a single turnover laser flash, measured in the absence and presence of NQNO, was not affected by prereduction with NADPH–Fd (panel A vs panel B of Figure 3). This shows that there can be at most only a small fraction of b_6–f complexes in which both hemes are reduced by NADPH–Fd, so that heme b_n alone is reduced by NADPH–Fd in almost all complexes. In addition, the peak of the spectrum of heme b_n reduced by NADPH was found to be at a shorter wavelength than that of heme b_p, obtained by subtracting the lower from the upper spectrum in Figure 2A, in agreement with Nitschke et al. (1988).

Inefficient electron transfer from heme b_n to heme b_p would not be expected from thermodynamic considerations since their E_m values are similar and the E_m of the $NADP^+$/NADPH couple (E_{m7} = –0.32 V) is much more negative. The possibility that a structural barrier to electron transfer between hemes b_n and b_p was responsible for the half-reduction by NADPH–Fd was examined through the effect of a lipophilic redox mediator on the extent of cytochrome b_6 reduction by NADPH–Fd. The E_m of this mediator (1,4-naphthoquinone, E_{m7} = 65 mV, E_m = +15 mV at the experimental pH, 7.8) was chosen to be slightly more positive than that of cytochrome b_6, so that in the oxidized state it could readily accept electrons from heme b_n and, when reduced, be able to reduce heme b_p. The extent of cytochrome b_6 reduction by NADPH–Fd was increased significantly in the presence of 1,4-NQ (compare trace b with traces a and c of Figure 4A). The E_m of the redox mediator may be critical in mediating electron transfer from heme b_n to heme b_p, since the addition of 1,2-NQ (E_{m7} = +135 mV) caused little or no change in the amplitude of the reduction by NADPH–Fd (compare trace b with traces a and c of Figure 4B).

Site of Action of NQNO. In analogy to the proposed action of antimycin A in chromatophores and mitochondria, the effect of NQNO on cytochrome b_6 reduction has been attributed to an inhibition of heme b_n oxidation (Selak & Whitmarsh, 1982). The increase in amplitude of the flash-induced reduction of cytochrome b_6 caused by addition of NQNO also occurred, however, when heme b_n as well as any quinone acceptor was prereduced by the NADPH–Fd (cf. Figure 3A,B). The same result was obtained at slower repetition rates (i.e., 0.033 Hz), assuring that heme b_n was reduced before each flash. In fact, heme b_n was reduced by NADPH in ~100 ms, as judged by the response using closely spaced flashes (see below, Figure 6). Thus, NQNO caused an increase in the amplitude of cytochrome b_6 reduction through an effect on heme b_p.

In the presence or absence of NQNO, the rate and extent of reoxidation following the flash-induced reduction were larger

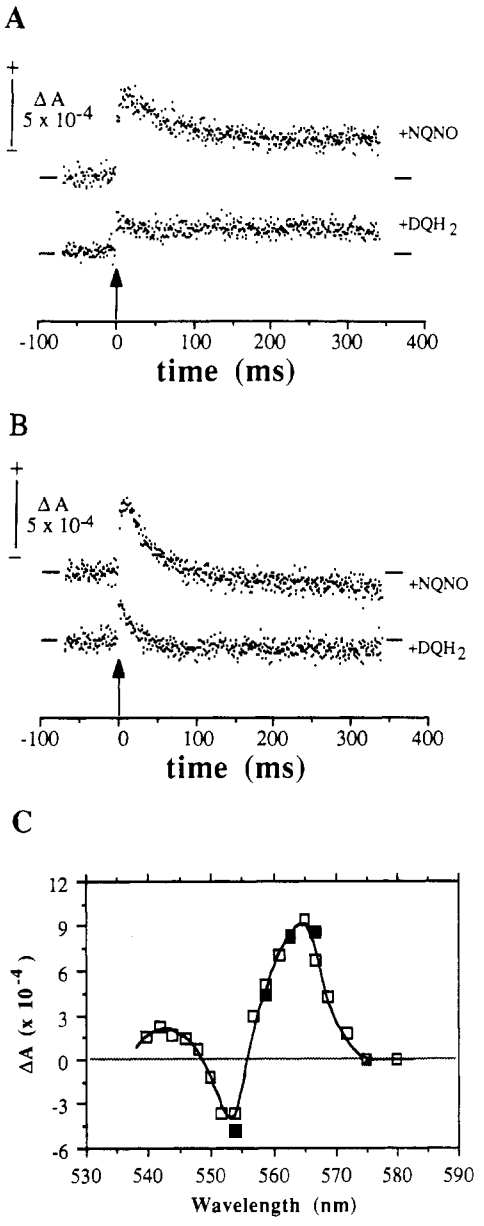

FIGURE 3: Effect of NADPH–ferredoxin on the amplitude of flash-induced reduction of cytochrome b_6. Reduction in the absence (A) and presence (B) of NADPH. Chloroplasts suspended at 40 μg of chl/mL, 1-cm measuring beam path length, in the presence of 4 μM DCMU, 5 μM gramicidin, 500 μM duroquinol, 5 μM ferredoxin, 1 μM NQNO when present [upper traces of (A) and (B)], 5.5 mM glucose, and 160 units glucose oxidase. Prereduction in (B) was achieved by addition of 1 mM NADPH. Temperature in cuvette, 20 °C. Traces are an average of 60 events, measured at 563 nm relative to a reference wavelength of 572 nm. Hash marks on the two sides of the recordings indicate the position of the original base line. (C) Spectrum of flash-induced absorbance change in cytochrome α-band region in the presence of Fd and NQNO and in the absence (□) and presence (■) of NADPH.

by approximately a factor of 4–5, with $t_{1/2} \approx 30$ ms (Figure 3B), when heme b_n was prereduced by NADPH–Fd. The effective $t_{1/2}$ for the oxidation in the absence of NADPH (Figure 3A) was >100 ms. The reoxidation is seen in Figure 3B to extend below the original base line. This has been observed only when heme b_n is initially reduced by NADPH, and was inferred to indicate oxidation of both hemes opposed by NADPH reduction of heme b_n. Since the faster reoxidation in the presence of NADPH–Fd occurs in the presence and absence of NQNO, the stimulation of the amplitude of b_6 reduction by NQNO is not necessarily a result of blocking reoxidation of heme b_p.

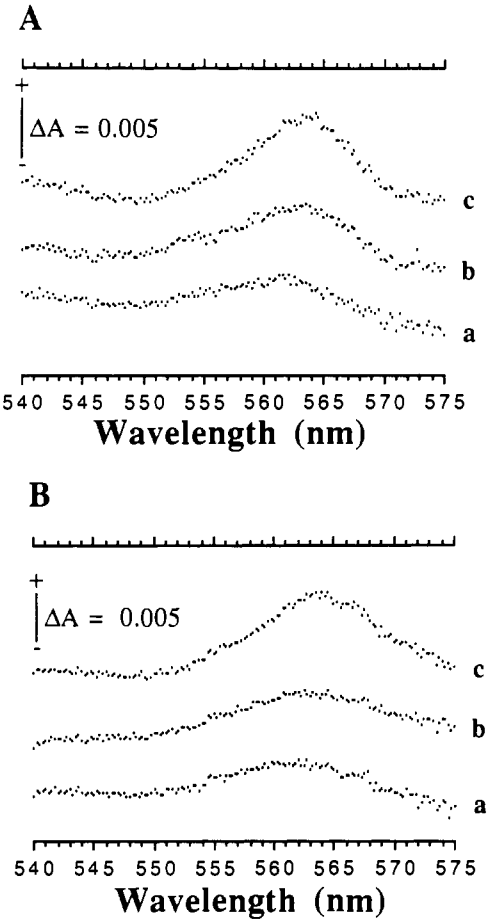

FIGURE 4: Effect of lipophilic redox mediators on the reduction of cytochrome b_6 by NADPH–Fd. (A) and (B): in the lower traces (a), 0.7 mM NADPH was added after preincubation in ferredoxin; in (c), full reduction of cytochrome b_6 was obtained by the addition of dithionite and anthraquinone; in trace (b), panel (A), 50 μM 1,4-naphthoquinone (E_{m7} = +60 mV) was added after preincubation with NADPH–Fd; in trace (b), panel (B), 50 μM 1,2-naphthoquinone (E_{m7} = +135 mV) was added after preincubation with NADPH–Fd. Conditions otherwise as in Figure 2.

The spectrum of the flash-induced reduction of cytochrome b_6 in the presence of NQNO is similar to that obtained by chemical and enzymatic reduction (compare Figure 3C with Figures 2A and 4). Spectral data obtained in the presence of oxidized ferredoxin (open symbols) and ferredoxin reduced by NADPH (closed symbols) were not distinguishable. The maximum amplitude of the reduction induced by a laser or xenon flash corresponded to 0.60 ± 0.09 heme/600 Chl (nine trials), under conditions where the laser flash was shown to be saturating by a linear correlation between the amplitude of cytochrome photoreduction and the chlorophyll concentration (data not shown).

Double and Multiple Flash Reduction of Cytochrome b_6. The Q cycle model involving interheme transfer implies that the two hemes of cytochrome b_6 should be reducible by successive light flashes if their reoxidation is completely inhibited by NQNO. The extent of reduction by the second or successive flashes should be greater, ultimately reaching twice the amplitude, than that of the first [as obtained with antimycin using b–c_1 complex (Hladik et al., 1987)]. Alternatively, if NQNO is a weak inhibitor, one might argue according to the Q cycle that there should be a binary oscillation in the pattern of flash reduction. Every second flash could lead to a more thermodynamically facile reduction of quinone through heme b_n. The faster oxidation of cytochrome b_6 reduced by a single flash after prereduction of heme b_n (Figure 3B) might

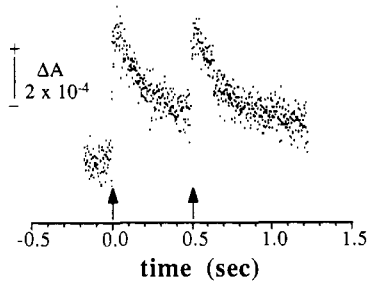

FIGURE 5: Two-flash reduction of cytochrome b_6. Laser flashes were given 0.5 s apart; successive measurements were at a 10-s interval, or 0.1 Hz. The first flash output had a discharge voltage of 18 kV, higher than needed for saturation, in order to provide sufficient output voltage, 13 kV, on the second flash. Cuvette temperature, 9 °C. Chloroplasts were suspended anaerobically (described above) at a concentration of 40 μg/mL, with the addition of 4 μM DCMU, 5 μM gramicidin, 5 μM ferredoxin, 500 μM DQH_2, and 1 μM NQNO.

be interpreted in this way. The effect of two successive saturating laser flashes (Figure 5), and of five xenon flashes (Figure 6), on the amplitude of cytochrome b_6 reduction was tested in the presence of NQNO and at 9 °C to slow the reoxidation (Figure 5), conditions that should allow the maximum cytochrome reduction. One heme (actually 0.6) was reduced by the first laser flash (Figure 5). When the second flash was delivered after a 0.5-s interval, the heme reduced by the first flash had been half-oxidized. Thus, it might be expected that reduction of the second heme would lead to an amplitude 1.5 times that achieved with the first flash. Alternatively, if NQNO is a weak inhibitor, binary oscillations would be expected in a Q cycle. However, the level of reduction caused by the second flash and the reoxidation kinetics were virtually identical. The intensity of the xenon flash was on the threshold of saturation. Thus, the amplitude of the response on the first flash was sometimes (Figure 6A) slightly (10–20%) below that of subsequent flashes. When the difference occurred between the first and second flashes, it could be determined by comparison with the laser flash that the response to the second flash corresponded to saturation (data not shown). It was not possible to obtain an increase in the amplitude of b_6 reduction on successive flashes when the xenon flash number was extended to five. The amplitude and reoxidation kinetics of the five flashes neither indicated a tendency toward reduction of a second heme upon successive flashes nor a binary oscillation in amplitude and rate. A similar result has been obtained by Rich et al. (1987). As in the case of the single-flash experiment (Figure 3A,B), the presence of NADPH did not affect the amplitude of the photoreduction (Figure 6B) although the reoxidation kinetics were faster as in Figure 3B. The result of the multiflash experiment carried out with duroquinol but in the absence of NQNO was that the first flash reduced 0.2 heme, as in Figure 3A,B, and the total reduction by the next four flashes was 0.0–0.1 heme (data not shown).

Discussion

Redox and Spectral Properties of Cytochrome b_6. The redox and spectral (visible and EPR) properties of the chloroplast cytochrome b_6 are much more homogeneous than those of the analogous cytochrome in mitochondria and photosynthetic bacteria. The difference between the E_m values ($\Delta E_m \lesssim 50$ mV) and reduced α-band maxima ($\Delta\lambda_m \approx 0.6$ nm) of cytochrome b_6 is smaller than the readily distinguishable differences of $\Delta E_m = 150$ mV and $\Delta\lambda_m = 4$–5 nm for the b cytochromes of the bacterial and mammalian mitochondrial b–c_1 complexes. Redox titrations fit reasonably well by a single component have been obtained of cytochrome b_6 in chloroplasts

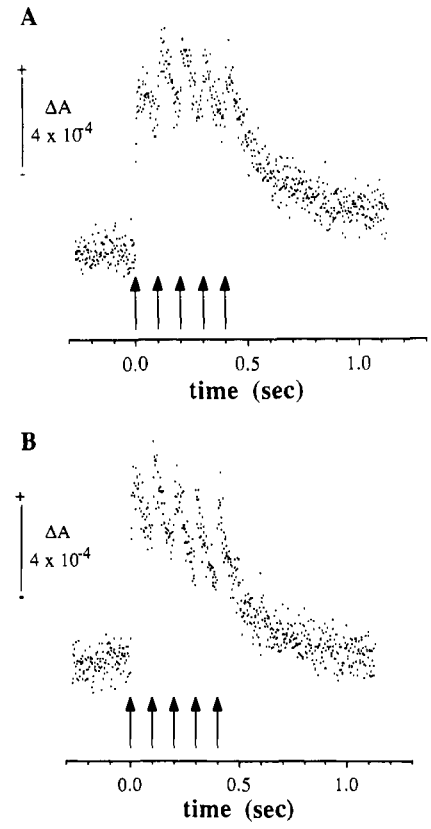

FIGURE 6: Multiple flash reduction of cytochrome b_6 in the absence (A) and presence of NADPH (B) that reduced heme b_n in the dark. Xenon flashes were spaced at 0.1 s within a train, while repeated flash trains were spaced 10 s apart. Temperature 20 °C. Conditions otherwise as in Figure 5.

(Böhme & Cramer, 1973; Böhme, 1976; Rich & Bendall, 1980; Girvin & Cramer, 1984; Bergström, 1985) and in isolated cytochrome b_6 from spinach chloroplasts at high pH (Hurt & Hauska, 1983). The ΔE_m of the cytochrome b_6 hemes is small enough, and the E_m of heme b_n sufficiently negative, that the thermodynamics for interheme electron transfer (i) are less favorable for the $b_p \rightarrow b_n$ reaction compared to the situation in photosynthetic bacteria and mitochondria and (ii) do not disfavor electron transfer from b_n to b_p, particularly if the source of electrons is NADPH. The situation is complicated by the known lability of one of the E_m values of cytochrome b_6. It can shift negatively by ~100 mV after illumination with steady light in the presence of high concentrations of FCCP or NH_4Cl (Böhme & Cramer, 1973). These conditions were not used in the present experiment, and there was no indication of cytochrome E_m lability in the titrations. However, this lability and the uncertainty of $\Delta E_m = 50$ mV between the two hemes do not exclude the possibility that the explanation for selective reduction of heme b_n by NADPH–Fd could be a thermodynamic barrier between heme b_n and heme b_p, although we think that the large barrier more likely arises from steric factors because of the redox mediator experiment (Figure 4) and because b_p reduction by NADPH–Fd in the absence of such mediators is not observed over a time scale of minutes.

Mode of Action of the Inhibitor NQNO. The observation that NQNO caused a similar increase in the amplitude of b_6 flash reduction after reduction of heme b_n and its putative quinone acceptor by NADPH–ferredoxin implies that the observable effect of NQNO is on the environment of heme b_p. It must act at a site distinct from that of DBMIB since it does not inhibit noncyclic electron transport at concentrations that stimulate cytochrome b_6 reduction (Jones & Whitmarsh,

1988). The sites of action of these two inhibitors may be in different niches in a "quinone pocket" near heme b_p. Much of the previous evidence on the involvement of interheme electron transfer from heme b_p to heme b_n in the mechanism of electrogenic H^+ translocation was based on the stimulatory effect of NQNO on the cytochrome b_6 photoreduction that suggested an analogy to antimycin A acting to inhibit oxidation of heme b_n. The present data imply, however, that NQNO enhances the flash reduction of heme b_p. The effect of NQNO on heme b_p might arise from its blocking a fast oxidation of heme b_p that could occur in the absence of NQNO. However, the acceleration of the cytochrome b_6 reoxidation when heme b_n is reduced would imply that the oxidation block could be only partial. Alternatively, the effect of NQNO might arise from an interaction of NQNO with the cytochrome and, in particular, elevation of the E_m of heme b_p. The analogue HQNO (20 μM) increased the E_m of both hemes by $\sim$100 mV (Clark & Hind, 1983). There are also other effects of NQNO that may be important. At the 1 μM concentration used in these experiments, NQNO inhibits the rate of cytochrome *f* reduction, and the rate and amplitude of the slow electrochromic phase, by approximately 2-fold (Selak & Whitmarsh, 1982; Moss & Rich, 1987).

Question of Interheme Electron Transfer. Reduction of less than one heme by multiple closely spaced saturating flashes, the absence of an indication for either reduction of two hemes or binary oscillations in amplitude or oxidation kinetics following such flashes, and an undiminished amplitude of flash-induced reduction after enzymatic reduction of heme b_n, imply that it is only heme b_p that is reduced by the flash. It is assumed that the mechanism of heme b_p reduction is the same oxidant-induced reduction as that proposed in the Q cycle model. The quinone binding site that serves for reduction of heme b_p by $PQ^{\bullet-}$ can also serve in the green alga *Chlorella* as the site for DNP-INT-sensitive oxidation of prereduced cytochrome b_6 (Delosme et al., 1987). The use of this pathway for the oxidation of heme b_p would be consistent with the conclusion that the electrogenic step under reducing conditions does not arise from interheme transfer (Girvin & Cramer, 1984). The inability to reduce more than one (actually, 0.6) heme by repetitive flashes has been observed previously by Rich et al. (1987), who considered three possible explanations: (i) a shift to high E_m of heme b_p, (ii) lack of interheme transfer, and (iii) rapid oxidation of the doubly reduced b_6 at heme b_n by a quinone competing with NQNO. The present data argue against a site of action of NQNO near heme b_n. The increase in reoxidation rate observed in the presence of NADPH–Fd and NQNO implies that the latter is a weak inhibitor, as discussed above and in agreement with Rich et al. (1987), and should not preclude observation of reduction of both hemes by double or multiple flashes. In contrast, it has been concluded that interheme electron transfer occurs in the isolated cytochrome b_6–*f* complex (Nitschke et al., 1988).

Photosystem I Cyclic Phosphorylation: Function of Heme b_n. A role of heme b_n in PS I cyclic electron transport is suggested by the ferredoxin requirement for its reduction by NADPH and the increase in the rate of reoxidation after a flash when heme b_n is poised in the reduced state by NADPH–Fd (Figure 3B). An increase in the amplitude of cytochrome b_6 reduction by ferredoxin has been demonstrated under continuous illumination, where it was inhibited by ferredoxin-specific antibody (Böhme, 1977). The cyclic pathway utilizing the transmembrane arrangement of the two b_6 hemes could be accomplished by oxidant-induced reduction of heme b_p linked to ferredoxin-mediated reduction of heme b_n. A possible problem with this pathway is that the rate of dark reduction of heme b_n by NADPH–Fd is slow under conditions where heme b_p would be oxidized (O'Keefe, 1983). However, heme b_n is reduced relatively rapidly by NADPH–Fd when heme b_p has been reduced by a flash, as indicated by the faster rate of reoxidation after closely spaced flashes (Figure 6, panel B vs panel A) that is diagnostic of prereduction of heme b_n (Figure 3, panel B vs panel A). The significance of slow reoxidation of ferredoxin relative to flash-reduced b_6 noted by de Wolf et al. (1988) is difficult to assess since the ratio of ferredoxin to b_6 turnover was 10:1.

On the Mechanism of H^+ Translocation in the b_6–f Complex. (a) Nonelectrogenic Pathway Associated with PS I Cyclic Phosphorylation. If cytochrome b_6 participates in PS I cyclic phosphorylation, the cytochrome may have a role in transferring H^+ to bound quinone in the same way that protonated amino acid residues of the D1–D2 complex serve as intermediates in protonation of the quinone at the Q_B binding site in PS II (McPherson et al., 1988). Then, the pH dependence of the E_m of cytochrome b_6 (Figure 1E) would be an indicator of a redox-linked protonation–deprotonation event associated with H^+ transfer.

(b) Electrogenic H^+ Translocation. A basic question is whether electrogenic H^+ translocation and an $H^+/e^- = 2$ ratio in the b_6–*f* complex are obligatory for noncyclic electron transport at steady-state values of the ΔpH or $\Delta\tilde{\mu}_{H^+}$. If so, then the H^+/e^- ratio would be 3 for whole-chain electron transport, and the $ATP/2e^-$ ratio = 2. The observed maximum value of the $ATP/2e^-$ ratio is approximately 1.25 (Hosler & Yocum, 1985) under conditions of noncyclic electron transport, close to the value, 1.33, predicted if the H^+/e^- ratio = 1 and 2 for the b_6–*f* complex and whole-chain electron transport, respectively. A value of $H^+/e^- = 1$ for the b_6–*f* complex is also implied by the electrically neutral oxidation of PQH_2 in the steady state (Graan & Ort, 1983). In any case, one must consider possible mechanisms of electrogenic H^+ translocation under a small $\Delta\tilde{\mu}_{H^+}$: (i) A redox-linked pump associated with electron transfer from PQH_2 to the Rieske center has been proposed (Girvin & Cramer, 1984). (ii) Plastosemiquinone, $PQ^{\bullet-}$, could generate the slow electrogenic phase and provide the vehicle for H^+ uptake from the stromal side by diffusing across the bilayer from the p side to the n side of the quinone pocket (Wikström & Saraste, 1984; Girvin, 1985). This semiquinone model has the following problems: (a) although heme b_n provides the electron for the $PQ^{\bullet-} \rightarrow PQH_2$ transition in a Q cycle model, according to the present work there is no pathway to reduce it in noncyclic electron transport. Component "G" in *Chlorella* has been proposed to mediate reduction of heme b_n from a stromal reductant (Joliot & Joliot, 1988). (b) High concentrations of duroquinol can reconstitute electrogenic H^+ translocation in the absence of any PQ (Willms et al., 1988), so that any putative quinone pocket must be of low structural specificity. (iii) Alternatively, a small displacement of $PQ^{\bullet-}$ from the Rieske iron–sulfur center toward the "pocket" and/or heme b_p could provide the redox link to the pump, a semiquinone-gated H^+ pump (Joliot & Joliot, 1986). The electron required for reduction of $PQ^{\bullet-}$ to PQH_2, concomitant with uptake of $2H^+$, could be supplied by heme b_p in a dismutase reaction (Hartung & Trebst, 1985). However, this model would predict that the oxidation of heme b_p after a flash should display a two-flash periodicity, which has not been observed.

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Specificity of the Interaction of Amino- and Carboxy-Terminal Fragments of the Mitochondrial Precursor Protein Apocytochrome *c* with Negatively Charged Phospholipids. A Spin-Label Electron Spin Resonance Study†

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ABSTRACT: The contribution of the various regions of the mitochondrial precursor protein apocytochrome *c* to the interaction of the protein with phosphatidylserine dispersions has been studied with chemically and enzymatically prepared fragments of horse heart apocytochrome *c* and phospholipids spin-labeled at different positions of the *sn*-2 chain. Three amino-terminal heme-less peptides, two heme-containing amino-terminal fragments, one central fragment, and three carboxy-terminal fragments were studied. The electron spin resonance spectra of phospholipids spin-labeled at the C5 position of the fatty acid chain indicate that both amino-terminal and carboxy-terminal fragments of the apocytochrome *c* molecule cause a restriction of motion of the lipids, whereas the heme-containing peptides and protein have less effect. In addition, a second motionally more restricted lipid component, which is observed for apocytochrome *c* interacting with phosphatidylserine dispersions containing lipids spin-labeled at the C12 or C14 position [Görrissen, H., Marsh, D., Rietveld, A., & de Kruijff, B. (1986) *Biochemistry 25*, 2904–2910], was observed both on binding the carboxy-terminal fragments and on binding of the amino-terminal fragments of the precursor protein. Interestingly, even a small water-soluble peptide consisting of the 24 carboxy-terminal residues gave rise to a two-component spectrum, with an outer hyperfine splitting of the restricted lipid component of 59 G, indicating a considerable restriction of the chain motion. This suggests that both the carboxy- and amino-terminal parts of the protein penetrate into the center of the bilayer and cause a strong perturbation of the fatty acyl chain motion. The implications of these findings for the mechanism of apocytochrome *c* translocation across membranes are discussed.

Most mitochondrial proteins are synthesized on free ribosomes in the cytoplasm as precursors with an amino-terminal extension (Hay et al., 1984; van Loon et al., 1988). These precursor proteins first must bind to mitochondria and subsequently insert into or translocate across one or two mitochondrial membranes, depending on the specific intramitochondrial location of the mature protein. The information for targeting the precursor proteins to mitochondria and also for intramitochondrial sorting is contained within the amino-terminal presequences (van Loon et al., 1988). The known mitochondrial presequences do not exhibit significant sequence homology (von Heyne, 1986), which would be expected if receptor proteins are involved. Chemically synthesized mitochondrial presequences are able to penetrate spontaneously into phospholipid monolayers and bilayers (Roise et al., 1986; Epand et al., 1986; Tamm, 1986), and a strong correlation exists between the model membrane penetrating capacity of the presequences and their ability to direct passenger proteins into the mitochondria (Roise et al., 1988). This is consistent with an involvement of lipid in mitochondrial precursor protein import. In order to understand the actual molecular mechanism of translocation of a precursor protein across a membrane, we have extensively studied apocytochrome *c*, the heme-free precursor of cytochrome *c* [for review, see Rietveld and de Kruijff (1986)].

Cytochrome *c* functions as a part of the mitochondrial respiratory chain and is located at the outside of the mitochondrial inner membrane. The protein is synthesized in the cytoplasm on free ribosomes, in a precursor form, apocytochrome *c*, that unlike most mitochondrial precursor proteins contains no amino-terminal presequence (Matsuura et al., 1981). This property allows the chemical preparation of large amounts of an import-competent precursor protein (Fisher et al., 1973; Zimmerman et al., 1979). After synthesis, apocytochrome *c* has to be imported into the mitochondrion. The import pathway probably does not involve translocation through the aqueous pores in the outer mitochondrial membrane (Manella et al., 1987). From biochemical analysis of the import of apocytochrome *c* into mitochondria (Zimmerman

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