Resonance Raman Evidence for Low-Spin Fe^{2+} Heme a_3 in Energized Cytochrome *c* Oxidase: Implications for the Inhibition of O_2 Reduction†

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ABSTRACT: Resonance Raman (RR) spectra are reported for reduced submitochondrial particles (SMP) with excitation at 441.6 nm, where Raman bands of the cytochrome *c* oxidase heme *a* groups are selectively enhanced. Addition of ATP to energize the membranes induces the formation of a new band at 1644 cm^{-1} and partial loss of intensity in a band at 1567 cm^{-1}. These changes are modeled by adding cyanide to reduced cytochrome *c* oxidase and are attributed to partial conversion of cytochrome (cyt) a_3 from a high-spin to a low-spin state. This conversion is abolished by addition of excess oligomycin, an ATPase inhibitor, or FCCP, an uncoupler of proton translocation, and is reversed when the ATP is consumed. The observed spin-state conversion is attributed to the binding of an endogenous ligand to the cyt a_3 Fe atom. This ligation is suggested to be induced by a local increase in pH and/or by a global conformation change associated with the generation of a transmembrane potential. Since O_2 binding requires a vacant coordination site at cyt a_3, the ligation of this site must retard O_2 reduction and could thus provide a simple mechanism for energy-linked regulation of respiration. No changes in the RR spectrum were observed upon adding Ca^{2+} or H^+ to reduced cytochrome *c* oxidase. The cyt a_3 spin-state change associated with membrane energization is unrelated to the cyt *a* absorption red shift induced by adding Ca^{2+} or H^+ to cytochrome *c* oxidase.

Cytochrome *c* oxidase (cyt ox.) is one of nature's primary energy transducers (Wikstrom et al., 1981). This multisubunit protein traverses the inner mitochondrial membrane, accepting four electrons from the cytosolic protein cytochrome (cyt) *c* and transferring them to the matrix side of the membrane, where they are used to reduce O_2 to water. The O_2 reduction reaction consumes one proton per electron delivered to the matrix; the redox free energy generated by this reaction is used to pump an additional proton from the matrix to the cytosol. The resulting proton electrochemical potential ($\Delta\mu_{H^+}$) across the membrane drives the ATP-hydrolyzing proton pump (F_1F_0ATPase) backward, synthesizing ATP from ADP and P_i (Nicholls, 1982).

Research on cytochrome *c* oxidase has yielded insight into the steps involved in electron and proton transport (Gelles et al., 1986; Naqui et al., 1986; Palmer, 1987; Wikstrom, 1987). It is widely accepted that O_2 is reduced at a binuclear site, containing a heme group, cyt a_3, and a copper atom, Cu_B. The input of electrons from cyt *c* occurs via two other metal centers, cyt *a* and Cu_A. Proton pumping appears to be mediated by the low-potential redox centers, cyt *a* and/or Cu_A (Wikstrom & Casey, 1985; Babcock & Callahan, 1983; Gelles et al., 1986). Various lines of evidence support the view that the transfer of electrons from the low- to the high-potential sites of cyt ox. involves protein conformational changes; this facilitates the binding of exogenous ligands to cyt a_3 (Scholes & Malmstrom, 1986), perhaps by perturbing or breaking a bridging interaction between cyt a_3 and Cu_B (Thomson et al., 1985). The transport of electrons and protons is tightly coupled, and indeed Wikstrom (1981, 1987) has shown it possible to reverse the O_2 reduction process at least partially, by adding ATP to mitochondria; absorption spectral signatures of intermediates in the later phases of O_2 reduction were detected.

In the present work we explore the question of energy coupling in submitochondrial particles (SMP), using resonance Raman (RR) spectroscopy as a structure probe of the heme *a* chromophores in cyt ox. Our results indicate that energization of the inner mitochondrial membrane in SMP is accompanied by partial conversion of cyt a_3 to a low-spin form, implying ligation of the heme group by an endogenous ligand. Intriguingly, Woodruff et al. (1989) have recently found evidence for endogenous ligand binding during the photodissociation of bound CO from isolated cyt ox. Since the resulting six-coordinate heme is unavailable for binding O_2, our observation suggests a straightforward physical mechanism for the inhibition of O_2 reduction in energized mitochondrial membranes.

Experimental Procedures

Raman spectra were collected in a backscattering geometry with a triple monochromator (SPEX 1877) equipped with a diode array optical multichannel detector (PAR 1420), cooled to −22 °C to reduce dark current. A 2400 groove/in. grating was used with slits set at 4–6-cm^{-1} resolution. Excitation at 441.6 nm was provided by a He–Cd laser (Liconix 4240NB) and at 413.1 nm with a Kr^+ laser (Coherent Innova 100-K3); the power at the sample was ~25 mW or lower.

Samples were contained in an anaerobic Raman cell, equipped with a small stir bar to provide gentle stirring for SMP energization experiments. Degassed ATP solution was added under nitrogen to a stirred degassed sample which had been positioned in the Raman chamber. Stirring was stopped, to reduce the background, and Raman spectra were collected as a function of time; the accumulation time per spectrum was 2–5 min. The maximum spectral changes occurred ~10 min after ATP addition and lasted less than 5 min at 5 °C; the

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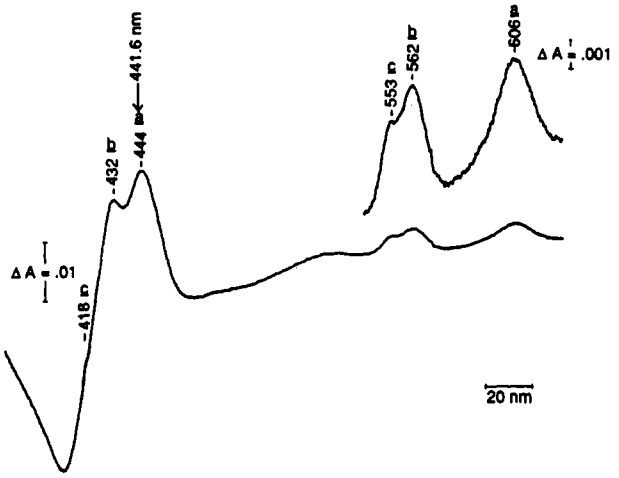

FIGURE 1: Room temperature difference absorption spectrum for reduced minus oxidized SMP (2 mg/mL) prepared as described under Experimental Procedures. Peaks are assigned for the different cytochromes *a*, *b*, and *c* present. The arrow marks the 441.6-nm RR excitation wavelength, in resonance with the B band of the reduced *a* cytochromes.

sample was cooled with a cold stream of nitrogen, and the temperature was monitored by a thermocouple probe inserted into the sample.

SMP (EDTA particles) were prepared by sonication of bovine heart mitochondria as described by Lee and Ernster (1967) and Lee (1979). The SMP pellets were suspended in 50 mM Tris buffer with 150 mM sucrose, pH 7.5, with a protein concentration of 20–25 mg/mL. Aliquots (160 μL) were stored in liquid nitrogen. They were thawed and diluted in Tris/sucrose buffer immediately before the Raman experiments. The reagents used were of the purest grade available commercially. Oligomycin (Sigma; mixture: 58% A, 30% B, 12% C), FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Aldrich], and other reagents were added as indicated in the figure captions. Conditions of maximum energization were estimated from the work of Lee et al. (1969) and adjusted experimentally to produce the maximum shift in the Raman bands (see Results). The room temperature difference absorption spectrum (dithionite-reduced minus oxidized) obtained with a Cary 2390 spectrophotometer (Figure 1) showed peaks characteristic of cyt *a* and cyt a_3 (444 and 606 nm), cyt *b* (432 and 562 nm), and cyt *c* (418 and 553 nm), (Lee et al., 1969).

Cytochrome *c* oxidase was prepared from fresh beef heart by the method of Li et al. (1987). Aliquots of concentrated suspension in 0.5% lauryl maltoside (CalBiochem) and 25 mM Tris buffer (pH 7.4) were stored in liquid nitrogen. Fully reduced cyanide-bound cyt ox. was prepared by anaerobic addition of degassed KCN solution to degassed cyt ox. that had been reduced with 8 mM $Na_2S_2O_4$. The absorption spectrum (442, 590, and 604 nm), monitored before and after the Raman experiments, showed no evidence of heme oxidation. For Ca^{2+} binding studies, aliquots of cyt ox. containing 5 mM EDTA were dialyzed against Hepes buffer (pH 7.4 or 6.7, for 36 h, three changes) to remove the EDTA and adjust the solution pH. Degassed $CaCl_2$ (50 mM) was added anaerobically to dithionite-reduced cyt ox. (140 μM), yielding a clear solution whose absorption spectrum showed no protein precipitation before or after the Raman experiments, using 413.1-nm excitation of a stirred solution, at 5–10 °C.

RESULTS

SMP Energization. Figure 1 shows a typical difference absorption spectrum for reduced minus oxidized SMP (Lee et al., 1969). The multiplicity of peaks reflects the multiplicity of cytochromes in the SMP; the *c* (418, 553 nm), *b* (432, 562 nm), and *a* (444, 606 nm) type cytochromes have progressively red-shifted bands (B, Q) due to increasing conjugation with peripheral substituents (vinyl, formyl). Laser excitation at 441.6 nm (arrow) produces selective excitation of the *a* cytochromes, without interference from the other chromophores in the membrane (Adar & Erecinska, 1979).

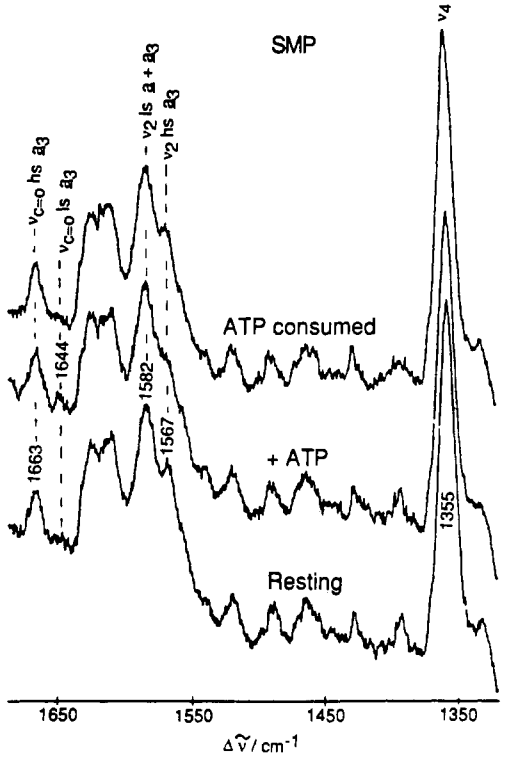

FIGURE 2: 441.6 nm excited RR spectra, at 5 °C, of (bottom) reduced SMP (13.3 mg/mL) with 0.3 μg of oligomycin/mg of SMP, 14.4 mM $MgSO_4$, and 3.8 mM $Na_2S_2O_4$. The middle spectrum had 12 mM ATP added anaerobically to generate a transmembrane proton gradient. For the top spectrum the sample was warmed to 16 °C for a few minutes, to use up the ATP, and cooled again to 5 °C. The 1355-cm^{-1} ν_4 band shows cyt *a* and cyt a_3 to be fully reduced in all species. Band assignments for high- and low-spin (hs, ls) cyt a_3 and cyt *a* (a_3, *a*) are indicated. There are overlapping contributions from other modes in the ν_2 region, especially ν_{37} and ν_{38}.

SMP are produced by sonication of mitochondria, which disrupts the inner membrane and forms closed vesicles containing both cyt ox. and the F_1F_0ATP synthetase (Lee & Storey, 1981). In these vesicles the matrix side is oriented outward, leaving the substrate binding sites accessible to the external solution. Anaerobic addition of ATP to fully reduced SMP leads to uptake of protons into the vesicles via ATP hydrolysis by the F_1F_0ATPase. Particles with energy-yielding efficiency close to that of mitochondria can be obtained (Lee et al., 1969).

Figure 2 shows 441.6 nm excited RR spectra in the 1300–1700-cm^{-1} region for SMP under different conditions of energization, at 5 °C. The entire spectrum of resting SMP is the same as that obtained with isolated reduced cyt ox., for which band assignments have been made (Choi et al., 1983; Babcock, 1988); assignments of bands that are important for the present study are indicated in the figure. The invariant 1355-cm^{-1} frequency of the strong ν_4 band is an indicator of the reduced redox state of both *a* cytochromes (Choi et al., 1983; Babcock, 1988) in all the SMP samples. The middle spectrum in Figure 2 shows the effect of energization of SMP upon the addition of ATP; a new band appears at 1644 cm^{-1},

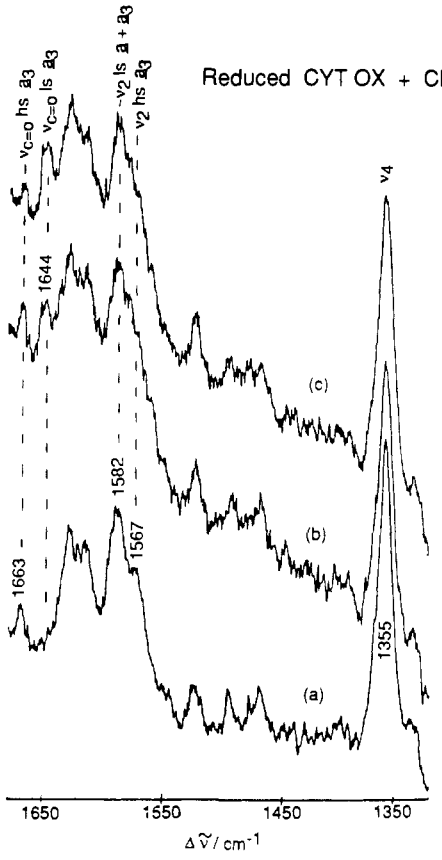

FIGURE 3: 441.6 nm excited RR spectra of reduced cytochrome *c* oxidase (184 μM) in the presence of excess cyanide (94 mM), under different laser powers, focus, and sample-stirring rates, which influence the degree of photoinduced deligation of CN^-: (a) 25 mW at the sample, tightly focused (fully deligated); (b) 7 mW, defocused beam, slow stirring (partially deligated); (c) same as (b) but fast stirring (mostly ligated).

and the 1567-cm^{-1} band loses intensity. These changes disappear upon warming the sample to 16 °C for a few minutes (top spectrum in Figure 2), due to the accelerated consumption of ATP by the ATPase. The changes are likewise abolished when excess oligomycin (4 μg/mg of SMP) is present during ATP addition. Oligomycin inhibits the F_1F_0ATP synthetase, thus preventing formation of the proton gradient. As expected, low concentrations of oligomycin are, however, necessary to establish this gradient, apparently serving to inhibit the uncoupled ATPase fraction which results from sonication (Lee & Ernster, 1968; Lee et al., 1969). We determined the optimum oligomycin and ATP concentrations empirically for maximum RR spectral changes (Figure 2 caption). Use of higher ATP concentrations (30 mM, plus 90 mM $MgSO_4$) with an independent batch of SMP gave the same spectral changes as shown in Figure 2, but 12 mM ATP was sufficient.

Finally, no RR spectral changes were observed upon addition of ATP in the presence of FCCP (5 μM), a weak acid uncoupler that short-circuits the transmembrane gradient by enhancing passive proton diffusion (Guerriere et al., 1976). Thus the evidence points conclusively to formation of the transmembrane proton gradient as the cause of the RR spectral changes.

Because of the weakness of the energy-linked spectral changes, we repeated these experiments several times with two independently prepared batches of SMP. The ATP-induced changes and their abolition by uncouplers were observed consistently.

Cyt Ox.–Cyanide Adduct. Figure 3 shows that the SMP spectral changes can be accurately modeled by simply binding cyanide to isolated cyt ox. in its fully reduced form. Cyanide binds to the vacant coordination site on cyt a_3, converting it from a five-coordinate high-spin to a six-coordinate low-spin heme (Ching et al., 1985). We find that this adduct is photolabile, however. The bottom spectrum in Figure 3 was obtained at a high incident laser power level, sufficient to fully photodissociate the bound cyanide and thereby produce the same spectrum as that of (cyanide-free) fully reduced cyt ox. An increasing fraction of cyt a_3 is bound to CN^- when the laser power is diminished and the beam is defocused (middle) and when the sample is stirred more rapidly (top), as evidenced by the growth of a band at 1644 cm^{-1} and a reduction in the intensity of the 1567-cm^{-1} band. These are the same changes as observed upon ATP addition to reduced SMP.

The intensity loss at 1567 cm^{-1} is best interpreted as a shift in the position of the ν_2 skeletal mode of heme *a*. This mode has been shown by Choi et al. (1983) to be sensitive to the porphyrin core size, and it shifts up 11–15 cm^{-1} when isolated Fe^{2+} heme *a* is converted from a five-coordinate high-spin to a six-coordinate low-spin complex (Choi et al., 1983; Babcock, 1988), due to the shorter low-spin Fe–N(pyrrole) bonds (Spiro, 1985). Thus, upon cyanide binding it is reasonable to expect ν_2 of cyt a_3 to shift up from 1567 to 1582 cm^{-1}, underneath the ν_2 band of the low-spin cyt *a*. This spectral region is complicated by overlapping contributions from other modes (especially ν_{37} and ν_{38}; Choi et al., 1983; Babcock, 1988) from the two heme groups, as evidenced by the significant intensity at 1567 cm^{-1} even in the top spectrum of Figure 3, corresponding to the mostly unphotolyzed cyanide adduct.

Spectral interpretation is clearer for the 1644-cm^{-1} band. This is the observed frequency for the formyl stretch of six-coordinate low-spin isolated heme $a^{2+}(NMeIm)_2$ in a hydrophobic environment (CH_2Cl_2; Babcock & Callahan, 1983; Choi et al., 1983). Thus the 1644-cm^{-1} band is assigned to the formyl stretch of cyanide-bound cyt a_3 (Ching et al., 1985). There is no interference in this case from cyt *a*, whose formyl stretch is at a much lower frequency, ~1610 cm^{-1} (in the complex envelope seen between 1600 and 1630 cm^{-1}; Babcock & Callahan, 1983). The lowered cyt *a* frequency is due to H-bond donation to the formyl group, as evidenced by its sensitivty to H/D exchange (Copeland & Spiro, 1986; Argade et al., 1986b; Babcock et al., personal communication). The heme group of cyt a_3, on the other hand, is known to be in a hydrophobic environment (Alben et al., 1982). For high-spin cyt a_3 the formyl stretching frequency is 1663 cm^{-1}, which is consistent with the 1660-cm^{-1} frequency observed for five-coordinate isolated heme a^{2+}(2MeIm) in CH_2Cl_2 (Choi et al., 1983; Babcock, 1988). The frequency difference between high- and low-spin heme *a* is attributable to back-donation of the low-spin iron d_π electrons to the porphyrin π^* orbitals, with which the formyl group is conjugated. As cyanide binds to reduced cyt ox, the 1663-cm^{-1} band is replaced by the 1644-cm^{-1} band (Figure 3). It can be seen from the spectra in Figure 3, however, that the molar intensity differs for the two bands, at the 441.6-nm excitation wavelength. The 1644-cm^{-1} low-spin band shows stronger resonance enhancement than the 1663-cm^{-1} high-spin band.

In the low-frequency region of the RR spectrum (not shown) a band at 214 cm^{-1} is assigned (Ogura et al., 1983) to the Fe–histidine stretching mode of reduced cyt a_3. This band appears upon photolysis of the cyanide adduct. Attempts to use its intensity to monitor the conversion of cyt a_3 to the low-spin form upon energizing SMP were frustrated, however, by the high background scattering at low frequencies in the SMP suspensions.

We note that the formyl band shifts up to 1666 cm^{-1} and ν_4 shifts up to 1368 cm^{-1} when CO (Argade et al., 1986a) or NO (Rousseau et al., 1988) binds to reduced cyt a_3, because the CO, a π-acid, competes with the porphyrin for the iron d_π electrons. This is why CN^- provides a more appropriate comparison with our SMP results, in which we observe a downshift of the formyl stretch and no shift of ν_4. These marker bands establish formation of a low-spin cyt a_3 complex with a non-π-acid ligand, presumably a protein side chain (see below).

Absence of a Calcium Effect. When mitochondria containing reduced cytochrome *c* oxidase are energized by increasing the phosphate potential, [ATP]/[ADP][P_i], a 1–2-nm red shift is observed in the absorption spectrum, which is largely attributable to cyt *a* (Wikstrom, 1972; Saari et al., 1980). A smaller red shift has been reported by Saari et al. (1980) when calcium is added to uncoupled mitochondria, and also to detergent-solubilized cytochrome *c* oxidase; for the latter, the effect could also be produced by lowering the pH. The Ca^{2+}-induced shift is diminished at lower pH, suggesting competitive interaction of Ca^{2+} ions and protons. A comparable red shift was found when calcium was added to the reduced bis(imidazole) complex of isolated heme *a*, but the shift was abolished if the heme propionate groups were esterified. Saari et al. (1980) inferred a common basis for all of these shifts and suggested specific protonation of the cyt *a* propionate groups as part of the mechanism of energy coupling.

Accordingly, we investigated the effect of adding Ca^{2+} to reduced cytochrome *c* oxidase at two different pH values, 7.4 and 6.7. In all cases the characteristic RR spectrum of reduced cyt ox. was observed. No effect could be detected, either of pH lowering or of adding calcium at a level greater than that sufficient to produce the absorption spectral shift. In particular, the specific RR changes seen upon SMP energization or upon cyanide addition to reduced cyt ox. were not observed.

It is not unexpected that absorption and RR spectra are responsive to different influences. Shifts in absorption bands reflect changes in the energy difference between ground and excited electronic states while shifts in Raman frequencies reflect alterations in the ground electronic state structure. Raman *intensities* do reflect the excited-state properties, but if a small absorption band shift produces a uniform intensity change in all Raman bands, this would be difficult to detect without careful measurements against an internal standard (not used in the present study). The interaction proposed by Wikstrom and co-workers to account for the absorption red shift, namely, binding of Ca^{2+} or H^+ by the heme propionate groups, might not produce a noticeable RR effect, since the propionate groups are not conjugated with the heme electronic system; the electrostatic effect might nevertheless be sensed sufficiently by the heme excited state to produce the small absorption red shift that is observed.

Conversely, there is reason to suppose that the formation of low-spin cyt a_3 during membrane energization does not significantly contribute to the observed absorption spectral shift, since CN^- addition to reduced cyt ox. produces *no* change in the Soret band and only a shoulder at 590 nm on the α-band (Ching et al., 1985). Thus the absorption and RR changes appear to reflect entirely different structural consequences of energization.

Discussion

Membrane Energization Induces a Low-Spin Component for Reduced Cytochrome a_3. The character of the RR spectral changes observed when ATP is added to coupled SMP, and their successful modeling with CN^- partially bound to isolated reduced cyt ox., leaves little doubt that a fraction of cyt a_3 is converted to a low-spin state. This conversion is definitively associated with the generation of a proton gradient by the F_1F_0ATPase, since it is eliminated by proton uncouplers (FCCP), or by inhibition of the ATPase (excess oligomycin), or simply by consumption of the ATP.

Only a minority population of the low-spin species is observed. From the relative intensities of the 1644- and 1663-cm^{-1} bands in reduced cyt ox. with and without bound cyanide (Figure 3), we estimate the degree of low-spin conversion to be on the order of 15% in the energized SMP. A likely reason for this low fraction is the difficulty of obtaining good energy coupling with the high SMP concentrations required for the Raman experiments, 60-fold higher than for the optical experiments which established the coupling conditions (Lee et al., 1969). Because some oligomycin must be added to inhibit the uncoupled ATPase, which is present as a contaminant in the sonicated particles, while higher concentrations of oligomycin inhibit the coupled ATPase, there is a narrow range of oligomycin concentration over which coupling can be observed. The higher the SMP concentration, the narrower this range, which we had to determine empirically, using the RR spectral changes as a guide. It is also possible that the degree of low-spin conversion is limited by the rate of dissipation of locally elevated pH on the external side of the vesicles (see below). Finally, it is possible that the low-spin adduct is photolabile in the laser beam, as is the CN^- adduct of reduced cyt ox. The maximum He–Cd laser power available was used, in order to obtain adequate signal/noise, and lowering the laser power to avoid photodissociation was not an option because of the limited duration of the energized species. Despite these experimental limitations, the spectral signature of low-spin cyt a_3 conversion, as well as its abolition by excess oligomycin and FCCP, was clearly observable in repeated trials of both batches of SMP that were tested in this study.

Cytochrome a_3 Ligation. In its reduced form cyt ox. contains a low-spin heme in cyt *a* and a high-spin heme in cyt a_3. The cyt a_3 Fe atom is five-coordinate, the fifth ligand being provided by a protein histidine side chain, as has been demonstrated by the identification of the iron–histidine stretching vibration in the RR spectrum (Salmeen et al., 1978; Ogura et al., 1983) and by EPR studies on ^{15}N-labeled yeast oxidase (Stevens & Chan, 1981). Binding of a sixth ligand with sufficient ligand field strength produces a low-spin heme. Conversely, identification of a low-spin cyt a_3 spectral component in energized SMP is prima facie evidence for binding of a strong-field sixth ligand. (Low-spin five-coordinate Fe^{2+} hemes do exist, but only when the ligand is a strong π-acid, such as CO or NO. As noted earlier, these ligands induce characteristic shifts in RR marker bands which would have been detected in our spectra. When bound to a single imidazole or any other ligating side chain available in a protein, the heme is high spin.) The midpoint potential of cyt a_3 has been estimated to decrease by 220 mV when mitochondria are energized (Lindsay & Wilson, 1972), indicating that the Fe^{3+} form becomes more stable in the energized state. This is the expected effect of adding a strong donor ligand to the Fe coordination sphere.

The only candidates for exogenous ligands in the SMP suspensions are water or hydroxide. These are both weak-field ligands and are not expected to bind Fe^{2+} strongly. A bis(hydroxide) Fe^{2+} heme adduct can be prepared in solution and is high spin (Parthasarathi et al., 1987). No model compound with imidazole and water or hydroxide bound simultaneously

to Fe^{2+} heme is available for comparison. Therefore, it cannot be entirely excluded that special features of the cyt a_3 pocket, e.g., appropriate disposition of a charged group, might stabilize a hydroxide complex, which might conceivably be low spin. It seems more likely, however, that the protein provides the sixth ligand. Possible candidates are side chains of histidine, cysteine, methionine, or lysine, all of which are expected to form low-spin six-coordinate complexes when the fifth ligand is histidine. Serine, threonine, and tyrosine are also candidates but are weaker field ligands and are more likely to form a high-spin complex. On the basis of an analysis by Holm et al. (1987) of invariant residues in the sequence of cyt ox. subunits I and II, it would appear that the available ligands (once those required for binding the four metal centers are taken into account) are four Met, three His, and two Lys residues; these are in subunit I, which is where the cyt a_3–Cu_B binuclear site is predicted to bind. The two invariant Cys and two invariant His residues in subunit II are presumably involved in binding Cu_A.

Recently, Woodruff et al. (1989) have made the arresting observation that the transient absorption and RR spectra of reduced cyt ox. immediately (<30 ps) after photolysis of bound CO show evidence for ligated cyt a_3, which then relaxes ($t_{1/2} \sim 1$ μs) to the equilibrium unligated cyt a_3. The transient species is suggested to result from binding of an endogenous ligand when the CO photodissociates from cyt a_3 and binds to the nearby Cu_B (Woodruff et al., 1989). This study provides independent evidence for the availability of an endogenous ligand to reduced cyt a_3.

Role of Membrane Energization. How might membrane energization induce the binding of a distal protein ligand to the cyt a_3 Fe atom? A global conformation change linking the proton pumping and O_2 binding activities provides a likely pathway. An allosteric conformation change has been proposed (Scholes & Malmstrom, 1986) to account for the observation that the rate of ligand binding to oxidized cyt a_3 is greatly increased upon reduction of cyt a and/or Cu_A, which are implicated in proton pumping (Wikstrom & Casey, 1985; Babcock & Callahan, 1983; Gelles et al., 1986). In this view, a protein motion linking the input and output sides of cyt ox. renders the cyt a_3 heme group more accessible to exogenous ligands, perhaps by releasing an endogenous ligand which may be present in the resting (oxidized) enzyme (Thomson et al., 1985; Powers & Chance, 1985). A similar mechanism could account for the energy-linked high- to low-spin conversion of cyt a_3 in the reduced form.

An additional possibility is that a potential endogenous ligand, e.g., His or Lys, becomes deprotonated upon formation of the energy-linked proton gradient. A deprotonation mechanism is suggested by RR studies of reduced cyt ox. showing that the cyt a_3 heme goes low spin when the pH is raised above 8.5–9 (Callahan & Babcock, 1983; Sone et al., 1986). If limited alkalinization is undertaken, the pH effect is reversible (Sone et al., 1986). The net effect of the proton circuit accross the mitochondrial inner membrane is an increase in pH on the matrix side, where O_2 is reduced (Nicholls, 1982). This pH increase might be sufficient to deprotonate a distal ligand and induce binding to cyt a_3. However, SMP are "inside out", with the cyt a_3 side of the membrane in contact with solution outside the vesicle; the solution volume is too large for the pH to be significantly altered by ATP hydrolysis. It is not impossible, however, that the local pH in the vicinity of cyt a_3 is increased upon membrane energization and that equilibration with the external solution is slow on the time scale of the RR experiment. The spectral change associated with low-spin cyt a_3 formation represents a small fraction of the total cyt a_3 and lasts only for a few minutes; perhaps the loss of the spectral response actually reflects the dissipation of the local pH elevation.

Respiratory Regulation. The energy released by O_2 reduction in mitochondria is partially converted to ATP production via the agency of the transmembrane proton gradient. The electron- and proton-transfer processes are coupled. The rate of respiration is high when the $[ATP]/[ADP][P_i]$ quotient is low, and it decreases when the latter increases. The means whereby this regulation is effected at the molecular level remains to be elucidated, however. Our observation of cyt a_3 ligation upon membrane energization suggests a straightforward mechanism, since the reduction of O_2 requires a vacant cyt a_3 coordination site. Binding of an endogenous ligand would block O_2 binding and would reduce the rate of reduction by the rate constant for the endogenous ligand dissociation. Thus the production of ATP via respiration would be subject to feedback inhibition via endogenous ligand gating of the O_2 binding site. Since there are several sites of energy-linked proton pumping, however, it is not expected that regulation of the respiration rate is limited to one locale. This may be an additional reason that only partial conversion to the cyt a_3 low-spin state is observed in energized SMP.

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Direct Electrochemistry of Protein–Protein Complexes Involving Cytochrome *c*, Cytochrome b_5, and Plastocyanin†

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ABSTRACT: The direct electrochemistry of the cytochrome *c*/cytochrome b_5 and cytochrome *c*/plastocyanin complexes has been investigated at edge-plane graphite and modified gold electrode surfaces, which are selective for one of the two components of the complex. Electrochemical response of one protein at an otherwise electrostatically unfavorable electrode surface was achieved in the presence of the other protein, and the calculated heterogeneous electron-transfer rate constant and diffusion coefficient were found to be in good agreement with the values determined previously from the electrochemistry of the individual proteins [Armstrong, F. A., Hill, H. A. O., & Walton, N. J. (1988) *Acc. Chem. Res. 21*, 407 and references therein]. A dynamic model of the protein–protein–electrode ternary complex is proposed to explain the promotion effect, and this model is supported by a study comparing the electrochemical responses of covalent and electrostatic cytochrome *c*/plastocyanin complexes. It is also suggested that the behavior of protein–protein complexes at electrode surfaces could be related to that of the complexes associated with biological membranes.

Electron transfer between redox proteins is essential to many metabolic processes in biological organisms. In vitro inter-protein electron-transfer reactions are believed to involve formation of a kinetically detectable precursor complex which is stabilized and oriented by electrostatic and hydrophobic interactions (Poulos & Kraut, 1980). Among redox protein complexes, those between cytochrome *c* and cytochrome b_5 (cyt *c*/cyt b_5)[1] and between cytochrome *c* and plastocyanin (cyt *c*/Pc) have been extensively studied, partly due to the availability of the crystal structure of each protein (Dickerson et

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[1] Abbreviations: EPG, edge-plane graphite; FPLC, fast protein liquid chromatography; pySSpy, 4,4′-dipyridyl disulfide; $(Cys\text{-}Glu)_2$, L-cystinylbis(L-glutamic acid); $(Lys\text{-}Cys\text{-}OMe)_2$, bis(L-lysyl-L-cystine methyl ester); cyt *c*/cyt b_5, cytochrome *c*/cytochrome b_5; cyt *c*/Pc, cytochrome *c*/plastocyanin.

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