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Steady-State Redox Behavior of Cytochrome *c*, Cytochrome *a*, and Cu_A of Cytochrome *c* Oxidase in Intact Rat Liver Mitochondria†

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ABSTRACT: We have examined the steady-state redox behavior of cytochrome *c* (Fe_c), Fe_a, and Cu_A of cytochrome *c* oxidase during steady-state turnover in intact rat liver mitochondria under coupled and uncoupled conditions. Ascorbate was used as the reductant and TMPD (*N,N,N',N'*-tetramethyl-1,4-phenylenediamine) as the redox mediator. After elimination of spectroscopic interference from the oxidized form of TMPD, we found that Fe_a remains significantly more oxidized than previously thought. During coupled turnover, Cu_A always appears to be close to redox equilibrium with Fe_c. By increasing the amount of TMPD, both centers can be driven to fairly high levels of reduction while Fe_a remains relatively oxidized. The reduction level at Fe_a is close to a linear function of the enzyme turnover rate, but the levels at Fe_c and Cu_A do not keep pace with enzyme turnover. This behavior can be explained in terms of a redox equilibrium among Fe_c, Cu_A, and Fe_a, where Fe_a is the electron donor to the oxygen reduction site, but only if Fe_a has an effective E_m (redox midpoint potential) of 195 mV. This is too low to be accounted for on the basis of nonturnover measurements and the effects of the membrane potential. However, if there is no equilibrium, the internal $Cu_A \rightarrow Fe_a$ electron-transfer rate constant must be slow in the time average (about 200 s^{-1}). Other factors which might contribute to such a low E_m are discussed. In the presence of uncoupler, this situation changes dramatically. Both Fe_c and Cu_A are much less reduced; within the resolution of our measurements (about 10%), we were unable to measure any reduction of Cu_A. Fe_a and Cu_A remain too oxidized to be in redox equilibrium with Fe_c during steady-state turnover. Furthermore, our results indicate that, in the uncoupled system, the (time-averaged) internal electron-transfer rate constants in cytochrome oxidase must be of the order of 2500 s^{-1} or higher. When turnover is slowed by azide, the relative redox levels at Fe_a and Fe_c are much closer to those predicted from nonturnover measurements. In presence of uncouplers, Fe_a is always more reduced than Fe_c, but in the absence of uncouplers, the two centers track together. Unlike the uninhibited, coupled system, the redox behavior here is consistent with the known effect of the electrical membrane potential on electron distribution in the enzyme. Interestingly, in these circumstances (azide and uncoupler present), Fe_a behaves as if it were no longer the kinetically controlling electron donor to the bimetallic center.

Cytochrome *c* oxidase is a biological energy transducer. The enzyme couples a redox reaction, the reduction of dioxygen to water by cytochrome *c*, to the production of an electrochemical proton gradient across the inner mitochondrial membrane. There appear to be two separate processes of energy conservation involved. One is essentially part of the redox reaction itself: The oxidation of one molecule of dioxygen to water requires four electrons and four protons. The enzyme is arranged in such a way that the electrons come from cytochrome *c* on the outside of the membrane, while the

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protons are taken up from the inner side of the membrane. Thus, the reduction of dioxygen to water produces a charge separation across the membrane equal to four electrical charges per dioxygen molecule, or an average of one proton per electron. The other energy conservation process, coupled to the same redox reaction, is a proton pump which translocates up to four additional charges (protons) across the membrane for every dioxygen molecule consumed (Wikström, 1977; Krab & Wikström, 1979).

Of four redox-active metal centers in the enzyme, two make up the site of oxygen reduction (Fe_{a_3} and Cu_B),[1] and two (Fe_a and Cu_A) function as intervening electron carriers between cytochrome *c* and the oxygen reduction site. The mechanism by which oxygen is reduced to water is now relatively well characterized (Chan et al., 1988; Hill et al., 1986; Malmström, 1982; Wikström et al., 1981; Wikström, 1981, 1987, 1988a), but very little is known about the function of Fe_a and Cu_A.

One popular idea is that either Fe_a or Cu_A (or both) is the site of energy transduction, an electron "gate" which controls electron flow coupling it to proton pumping. In such a scheme, the oxygen reduction site's only role in the proton pump mechanism would be that of electron acceptor [see reviews by Chan and Li (1990) and Krab and Wikström (1988)]. Alternatively, it has been suggested that Fe_a and Cu_A are simply electron carriers and that the active locus of energy transduction is the oxygen reduction site (Chance & Powers, 1985; Mitchell, 1987). The recent finding that the four one-electron steps in the oxygen reduction mechanism are unequally coupled to proton pumping (Wikström, 1989) is consistent with the latter idea. The actual mechanism could, of course, include elements of both of these schemes. At the present time, however, even the pathways of electron flow through the enzyme remain to be charted. There is structural data to indicate that Cu_A is the primary acceptor of electrons from Fe_c (Holm et al., 1987; Millett et al., 1983), but direct electron transfer from Fe_c to Fe_a cannot be ruled out, and the route(s) by which electrons reach the oxygen binding site during turnover is (are) still not established. Clearly, knowing the precise electron-transfer sequence would be an important step toward understanding how electron flow is coupled to proton translocation.

One way to approach this question is to follow the redox state of the metal centers spectroscopically during steady-state turnover. This approach has the disadvantage that it only gives a time-averaged picture of the system. Yet, such studies may help to exclude certain electron-transport models, and may provide the basic framework for acceptable models. A large number of studies of this kind have been reported, originally in mitochondria [e.g., see Chance and Williams (1955)], later in the isolated, detergent-solubilized enzyme [e.g., see Yonetani (1960)], and most recently with the enzyme reconstituted into vesicles (see below). The advantage of the mitochondrial and vesicular systems is that the redox reactions can be studied under conditions where there is an actual load (the electrochemical proton gradient) against which the reaction must do work. Over many years, the steady-state redox behavior of Fe_a and Fe_c has received a great deal of attention. Recently, several groups have studied the separate effects of pH, ΔpH, and $\Delta\Psi$ on the steady-state properties of these centers in reconstituted cytochrome oxidase vesicles (Moroney et al., 1984; Nicholls et al., 1988; Gregory & Ferguson-Miller, 1989). In contrast, the steady-state redox behavior of Cu_A has almost exclusively been studied in soluble enzyme systems where the effects of membrane potential cannot be seen [Wilson et al., 1975; Thörnström et al., 1988; but see Erecinska et al. (1974)].

Here we have studied the steady-state redox behavior of Cu_A in intact mitochondria and compared its behavior with that of Fe_a and Fe_c. After spectroscopic interference from the redox mediator TMPD was eliminated, we found that, in the mitochondrial system, Fe_a is less reduced during steady-state turnover than previously thought [e.g., see Wikström et al. (1976)]. In the coupled system, Cu_A behaves very much like Fe_c, but in the presence of uncoupler, the reduction level of Cu_A was lower than what we were able to resolve (about 10%).

Materials and Methods

Reagents. TMPD dichlorohydride was obtained from Fluka A.G., myxothiazol from Boehringer Mannheim, and rotenone from Sigma. FCCP was a gift of P. G. Heytler. Horse heart cytchrome *c* (type VI) was obtained from Sigma and used without further purification.

Preparation of Samples. Rat liver mitochondria, mitoplasts, and cytochrome *c* depleted mitoplasts were prepared by the method of Greenawalt (1974) with minor modifications. For mitochondria, a medium containing 0.25 M sucrose and 0.1 mM EGTA adjusted to pH 6.8–6.9 with Tris was used, while for mitoplasts a medium containing 70 mM sucrose, 220 mM D-mannitol, 2 mM HEPES, and 0.5 mg/mL BSA was used. The concentration of cytochrome aa_3 was determined, after solubilization in detergent, by using a millimolar absorptivity of 26.5 cm^{-1} at 605 minus 630 nm. Calculations of mitochondrial protein concentrations are based on a value of 0.14 mmol of cytochrome *c* oxidase/mg of protein (Wikström & Saraste, 1984).

Experiments were carried out in buffers containing either 10 mM HEPES, 10 mM MOPS and 10 mM Tris, 20 mM KCl and 200 mM sucrose, or 70 mM MOPS and 200 mM sucrose. Buffer pH was 7.2 unless otherwise noted. Unless otherwise indicated, the samples also contained the following additions (typical concentrations given in parentheses): EGTA (2 mM), rotenone (10 μM), myxothiazol (8 μM).

When called for, FCCP was used to uncouple the mitochondria. In the polarographic assay, 0.29 μM FCCP was used, and the highest uncoupled turnover rates were fairly consistent, even when the amount of mitochondria was varied. (0.15 μM FCCP, or about 1.4 nmol/mg of protein, was needed to uncouple fully at 3.5 mM TMPD and a mitochondrial cytochrome *c* oxidase concentration of 17 nM). In the spectroscopic samples, much larger amounts of mitochondria were used (typically, 1–2 μM cytochrome aa_3). It was impossible to measure the enzyme activity at spectroscopic concentrations. Extrapolating the FCCP concentration on the basis of the FCCP to protein ratio would have called for concentrations on the order of 10–20 μM FCCP. Rather than use such extreme concentrations, we titrated the effect of FCCP on the spectroscopic properties of the system at a fairly high concentration of TMPD (2.1 mM). The response of Fe_a to the addition of FCCP is not simple. At this TMPD concentration, small amounts of FCCP (0.01 nmol/mg of protein) caused the steady-state reduction level of Fe_a to fall from 24% to about 17%. Beyond this point, increasing the amount of FCCP caused reduction to increase again, leveling off at about 22%

[1] Abbreviations: BSA, bovine serum albumin; $\Delta\mu_H$, proton electrochemical potential difference; $\Delta\Psi$, electrical potential difference; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; E_m, midpoint redox potential relative to normal hydrogen electrode; E_h, redox potential relative to normal hydrogen electrode; Fe_a, (heme iron of) cytochrome *a*; Fe_{a_3}, (heme iron of) cytochrome a_3; Fe_c, (heme iron of) cytochrome *c*; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*',*N*'-tetramethyl-1,4-phenylenediamine; $TMPD^+$, oxidized form of TMPD; Tris, tris(hydroxymethyl)aminomethane.

(at 0.4 nmol of FCCP/mg of protein). (The level of Fe_c reduction decreased throughout this range.) FCCP at 3.5 μM (0.56 nmol/mg of protein) was used for the spectroscopic samples under high-turnover conditions (see figure captions).

Activity Measurements. Enzyme activity was measured polarographically using a Clark-type oxygen electrode (Yellow Springs Instrument Co. Inc.) and an amplifier built by the University of Pennsylvania Biomedical Instrumentation Group.

Spectroscopy. Routine optical spectra were recorded with a Shimadzu UV-3000 spectrophotometer. The data in Figure 2 were measured with this instrument and were digitized and recorded to computer by means of a DT2825 data acquisition card (Data Translation Inc.).

Other optical measurements (Figures 3–6) were made with a DBS-1 dual-wavelength spectrometer (University of Pennsylvania Biomedical Instrumentation Group). For measurements in the near-infrared, the following changes were made to the normal DBS-1 configuration: A photomultiplier with enhanced red sensitivity (type 9202A, Thorn EMI Electron Tubes Ltd.) was used. To ensure proper order selection by the monochromators, cutoff filters were placed in the measuring and reference beams between the monochromators and the chopper (measuring beam, 700-nm cutoff; reference beam, 750-nm cutoff). For measurements in the visible region, one of two photomultipliers (9202A or 9659QB, Thorn EMI) was used; 10-mm path-length glass optical cuvettes were used in all cases.

Spectroscopic Measurement in the Presence of $TMPD^+$. The oxidized form of the redox mediator ($TMPD^+$, Wurster's blue) accumulated during steady-state turnover, interfering with measurement of heme spectra (see Results). By use of conventional dual-wavelength spectroscopic methods (Chance 1954), this interference was minimized by choosing new reference wavelengths at which the absorptivity of $TMPD^+$ is the same as at the measuring wavelength. The wavelengths used were (approximately) 605 minus 620 nm for Fe_a and 550 minus 582 nm for Fe_c.

The reference wavelength was set empirically before each experiment in the following way: First, the measuring beam monochromator was set to the correct wavelength, and the reference beam monochromator was set to an approximate reference wavelength. Then the reference wavelength was tuned to minimize the absorbance jump produced when a small amount of $TMPD^+$ was added to buffer (or, in some cases, to a sample of mitochondria).

$TMPD^+$ for this purpose was made in one of two ways: (1) TMPD was oxidized by mitochondria. In some cases, TMPD was added to a mitochondrial sample in the spectrophotometer, and the production of $TMPD^+$ was followed in situ. However, the usual protocol was to add mitochondria (with rotenone and myxothiazol) to the TMPD, vortex the sample for a few minutes, spin the mitochondria down, and then to repeat this process for a second cycle. (2) Alternatively, $TMPD^+$ was made by adding solid ferricyanide to a solution of TMPD.

The actual reference wavelength settings were slightly variable (±about 1.5 nm), but in the text and figures, the numbers 620 and 582 nm are used throughout. For the experiments on azide-inhibited mitochondria, the monochromators were not tuned, but simply set to 605 minus 620.5 nm and 550 minus 582.5 nm. Measurements at 605 minus 630 nm indicate that some $TMPD^+$ does build up, even in the presence of azide, but that the error in the 605 minus 620.5 nm measurements (at 18 s^{-1}) would be less than 3%.

Fe_a was assumed to contribute 87% of the total reduced minus oxidized absorbance change at 605 minus 620 nm. This value is based on the saturating steady-state reduction level in our experiments with azide-inhibited mitochondria. This is in reasonable agreement with values obtained with cyanide, where the fully reduced minus oxidized absorbance change (at 605 minus 630 nm) is 89% of that in the absence of cyanide.

In the measurements of Fe_c reduction, no attempt has been made to compensate for the contribution of cytochrome c_1. Typical rat liver mitochondria contain 1.4 cytochromes *c* and 0.5 cytochrome c_1 for every cytochrome oxidase (Wikström & Saraste, 1984), and cytochrome *c* and cytochrome c_1 have approximately the same absorptivity at 550 nm (the cytochrome c_1 extinction is slightly smaller; Nicholls, 1976). Thus, reduction of cytochrome c_1 should account for about 0.26 of the total reduced minus oxidized absorbance change at 550 nm. However, this should not introduce any large errors into the results since cytochromes *c* and c_1 are thought to be in rapid equilibrium (Nicholls, 1976), and the E_m of cytochrome c_1 is only 5 mV higher than that of membrane-bound Fe_c (Dutton et al., 1970).

Steady-State Kinetic Measurements. Unless otherwise noted, experiments were carried out at 25 °C. Steady-state turnover in aerobic samples of mitochondria was initiated by adding, first, ascorbate (as a neutralized 2 M solution) and then TMPD (as an aqueous solution). The point-by-point determinations of absorbance during steady-state turnover as a function of TMPD concentration were made with the DBS-1, by following the absorbance continuously as the samples went from the oxidized state, through steady-state turnover, to full reduction when the oxygen in the sample was exhausted.

Steady-state turnover could be restarted, after anaerobiosis, by adding small volumes of a hydrogen peroxide solution (stock concentration, 200 mM or less), relying on the catalase activity of the mitochondrial preparation to rapidly convert the peroxide to oxygen and water. This could be repeated several times. In this way, it was possible to obtain measurements under both coupled and uncoupled conditions from the same sample. In all cases, care was taken not to exceed the limits of oxygen solubility by any large extent. Some of the spectra in Figure 2 were scanned during steady-state turnover. In cases where the duration of the steady state was too short for a full scan to be made, the spectra were acquired in segments, with steady-state turnover restored during each segment by the addition of hydrogen peroxide.

Data Handling. The redox potentials for the uninhibited coupled system were estimated by comparing calculated Nernstian curves to the data. In all other cases, redox potentials were estimated from redox levels at a single point in the curve. Nernstian $n = 1$ behavior was always assumed. All redox potentials are with reference to the normal hydrogen electrode.

In order to plot data with enzyme activity rather than TMPD concentration as the "independent" variable, it was necessary to interpolate the enzyme activity vs TMPD concentration data. This was done by fitting arbitrary polynomials to the data. In the case of the coupled data, two different polynomials were used, one for points below 0.22 mM TMPD and one for points above. In the case of the azide experiment, the activity data were interpolated manually, and the interpolated values were read into computer using a digitizing tablet.

Results

Ascorbate–TMPD System in Mitochondria. Steady-state turnover of cytochrome *c* oxidase has frequently been studied by using ascorbate as the ultimate electron donor and TMPD

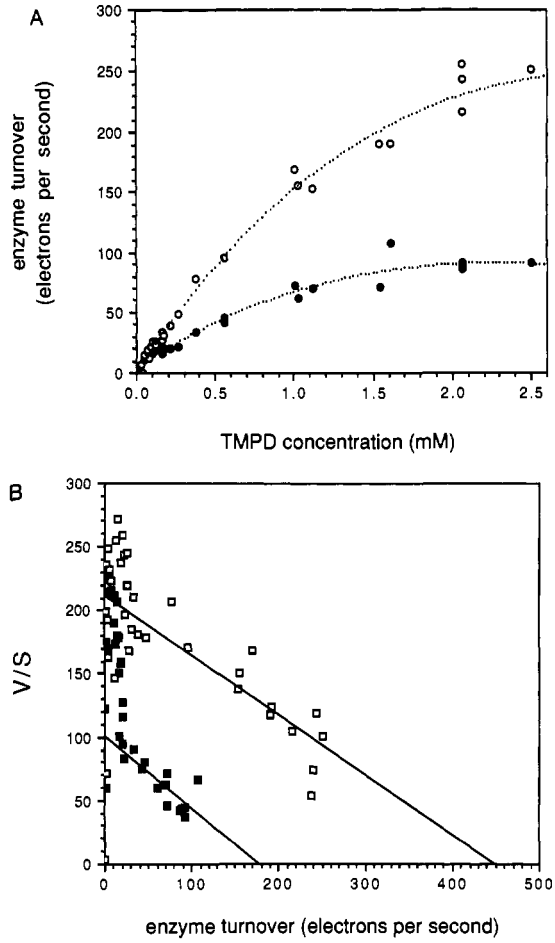

FIGURE 1: (A) Enzyme turnover as a function of TMPD concentration in coupled and uncoupled mitochondria: (●) without FCCP; (○) with FCCP. Enzyme activity was determined by measuring oxygen consumption polarographically. FCCP concentration, 0.29 μM. Dotted lines are polynomial fits to the data. Their only purpose is to interpolate so that enzyme turnover values can be used in subsequent figures (see Materials and Methods). (B) Eadie–Hofstee plot of activity data: (■) without FCCP; (□) with FCCP. The lines are linear fits to the points with activity greater than 25 s^{-1}.

as the electron mediator to cytochrome *c*. This system is well suited to the study of the enzyme in intact mitochondria since these organelles contain endogenous cytochrome *c* which can be reduced in situ by TMPD (Kimelberg & Nicholls, 1969; Hill & Nicholls, 1980). The remainder of the respiratory chain can be blocked with inhibitors. This simulates the natural activity of the cytochrome *c* oxidase segment of the respiratory chain and allows the rate of electron flow through the system to be modulated by the choice of TMPD concentration.

Activity Data. Figure 1A,B shows the rate of turnover of cytochrome *c* oxidase in mitochondria in the presence and absence of FCCP. In both the coupled and the uncoupled systems, the turnover kinetics are biphasic with respect to the TMPD concentration. The low-activity phases are not well enough resolved to evaluate quantitatively, but in the higher activity phases the V_{max} is about 180 (electrons per second) in the coupled system and about 450 in the uncoupled system. As the TMPD concentration is increased, the ratio of uncoupled to coupled activity increases rapidly to about 2.0 at about 150 μM TMPD, after which it increases much more slowly. The V_{max} for the uncoupled state is about 2.5 times that for the coupled state.

It is important to note that these parameters are based on the concentration of TMPD, and not cytochrome *c*, as substrate. Thus, the biphasic behavior here may or may not be related to the biphasic behavior of cytochrome oxidase with respect to cytochrome *c* concentration (see Discussion). Furthermore, the ratio of uncoupled to coupled rates is not directly comparable to the "respiratory control ratio" (RCR) generally used to characterize the quality of coupling in mitochondria. The RCR for the cytochrome oxidase reaction should be defined as the ratio of uncoupled to coupled activity *at constant ferrocytochrome c* (Fe_c^{2+}) *concentration* (Wilson et al., 1977). In the present experiment, when uncoupler is added, the level of Fe_c reduction drops dramatically (see below). With this taken into account, the true RCR of the system is much higher than 2.5, and considerable respiratory control is exhibited even at the lowest concentrations of added TMPD.

Effective Concentration of TMPD. In this paper, the dependence of a number of properties of steady-state turnover is correlated and compared. TMPD may be able to bind to the mitochondrial membrane, and, thus, the effective concentration of TMPD in a sample could be higher when less mitochondria are used. Different measurements often require different amounts of mitochondria, and there was no way to use a constant mitochondrial concentration in all cases. As an example, for the polarographic activity measurements, the mitochondrial concentration had to be varied at least 100-fold in order to cover the necessary range of turnover. In the spectroscopic samples, the range was only a factor of about 4.

In what follows, we have plotted measured parameters versus turnover velocity. In order to do this, we have used the curves in Figure 1A, which are polynomials fit to the activity data (see Materials and Methods), to calculate the enzyme activity from the TMPD concentration. In some cases, we have also included the scale of TMPD concentration for comparison.

Spectroscopic Interference by $TMPD^+$. In this reaction, TMPD cycles between its reduced and oxidized forms. The oxidized form ($TMPD^+$) accepts an electron from ascorbate, and the TMPD thus formed donates one electron to Fe_c (Dutton, 1978). TMPD is colorless, but $TMPD^+$ is an intensely colored dye known as Wurster's blue (Dawson et al., 1987), which can potentially interfere with observation of heme spectra.

When TMPD has been employed in spectroscopic experiments, the conventional strategy has been to use an excess of ascorbate in order to keep the mediator essentially in its reduced form (Kimelberg & Nicholls, 1969). However, our measurements on Fe_c and Fe_a in intact rat liver mitochondria showed that this approach would not work. When higher concentrations of TMPD were used to obtain high turnover rates, there was always a significant amount of $TMPD^+$ in the samples, enough to interfere with the spectroscopic measurement of Fe_a and, to a smaller extent, Fe_c. Similar problems have also been encountered before with submitochondrial particles (Sagi-Eisenberg & Gutman, 1979) and with the isolated reconstituted enzyme (Nicholls et al., 1988; Gregory & Ferguson-Miller, 1989). The accumulation of $TMPD^+$ at high concentrations of added TMPD is, however, small enough not to significantly upset the equality of added TMPD and true TMPD concentrations.

Figure 2a shows a difference spectrum of rat liver mitochondria during steady-state turnover, together with spectra of the reduced and oxidized states. In the reduced spectrum, absorption peaks arising from Fe_c^{2+} (550 and 520 nm) and Fe_a^{2+} (605 nm) can be seen ($Fe_{a_3}^{2+}$ contributes 10–20% of the

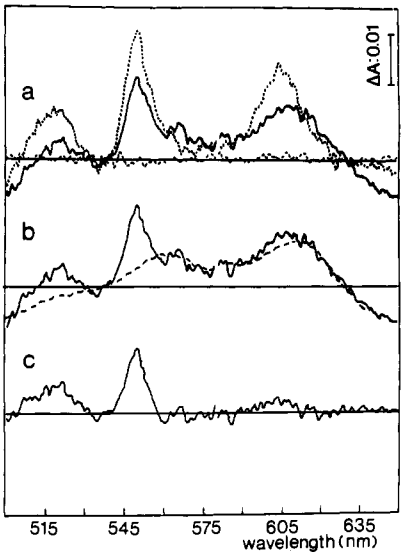

FIGURE 2: Spectra of mitochondria during coupled steady-state turnover of cytochrome *c* and cytochrome oxidase, showing accumulation of TMPD$^+$. (a) Mitochondria during steady-state turnover (solid line) together with spectra of reduced (dots) and oxidized (dashes, base line) mitochondria; (b) same steady-state spectrum together with a spectrum of TMPD$^+$ (dashes) arbitrarily scaled to match; (c) steady-state spectrum after subtraction of the TMPD$^+$ spectrum in (b). These dual-wavelength difference spectra were each acquired in two segments (with separate samples for each segment): for 650–580 nm, reference wavelength = 630 nm; for 580–500 nm, reference wavelength = 540 nm. TMPD concentration, 1.08 mM.

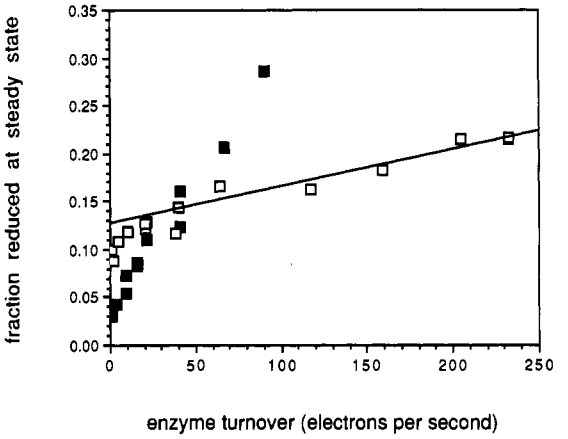

FIGURE 3: Fe_a: steady-state reduction levels as a function of enzyme turnover in coupled and uncoupled mitochondria. (■) Without FCCP; (□) with FCCP. In order to avoid interference from TMPD$^+$, Fe_a reduction was measured at 605 minus 620 nm (approximately); the data have been adjusted to take into account the contribution of Fe_{a_3} at these wavelengths (see Materials and Methods). FCCP concentrations for spectroscopic measurements: below 40 s^{-1}, 0.2–0.4 μM; above 40 s^{-1}, 3.5 μM. The line is a fit to all points in the +FCCP data with activity over 40 s^{-1}.

intensity at 605 minus 630 nm; Vanneste, 1966; Wikström et al., 1976). However, in the steady-state spectrum, the dominant contribution at 605 nm is clearly not Fe_a^{2+}, but TMPD$^+$. In Figure 2b, a spectrum of TMPD$^+$ is superimposed on the steady-state spectrum from Figure 2a to show their similarity. (Note that both the steady-state spectrum and the TMPD$^+$ spectrum exhibit a trough above 630 nm which is largely absent from the reduced spectrum.) Subtraction of the TMPD$^+$ spectrum from the steady-state spectrum gives the result shown in Figure 2c. With the TMPD$^+$ contribution removed, the remaining components of the steady-state spectrum can be seen to be Fe_c and Fe_a (but see below) and a small peak from a *b* cytochrome. The 605-nm peak is now very small. In light-scattering systems such as mitochondrial suspensions, Fe_a^{2+} has traditionally been measured as the 605 minus 630 nm absorbance difference (Chance, 1954). If we had used these wavelengths to measure Fe_a^{2+}, the TMPD$^+$ would have caused a gross overestimation. Fe_c^{2+} would have been overestimated as well, although to a smaller extent.

The steady-state concentration of TMPD$^+$ could be decreased somewhat by using high concentrations of ascorbate, but there was always significant interference even when ascorbate concentrations in excess of 50 mM were used (data not shown).

Thus, the presence of spectroscopically adverse concentrations of TMPD$^+$ in this system could not be eliminated, and it was necessary to find a way to measure the redox levels of Fe_c and Fe_a in the presence of the dye. In order to do this, we employed the classical method of dual-wavelength spectroscopy (Chance, 1954) but chose the reference wavelengths such that TMPD$^+$ would not contribute to the final measurement. The wavelength pairs used were 605 minus 620 nm for Fe_a and 550 minus 582 nm for Fe_c. (These reference wavelengths are approximate; the precise monochromator settings must be determined experimentally; see Materials and Methods.)

Steady-State Redox Behavior of Fe_a. Figure 3 shows the steady-state minus oxidized absorbance change at 605 minus 620 nm as a function of turnover velocity. The data have been scaled so that this value corresponds to the reduction level of Fe_a (assuming that Fe_{a_3} contributes 13% of the total reduced minus oxidized difference; see Materials and Methods). In coupled mitochondria, after a small initial jump, the reduction level of Fe_a is approximately a linear function of turnover velocity. The size of the initial jump varies from one batch of mitochondria to the next, but the slope of the dependence is reasonably consistent, and at a turnover rate of 90 e^-/s, the steady-state reduction level of Fe_a varies between 22 and 33% in different batches of mitochondria (see also Figure 5). In uncoupled mitochondria, there is clear biphasic behavior: At very low turnover velocity (less than 20 μM TMPD), the absorbance change quickly rises to about 12% ("phase I"). Beyond that point, it increases linearly, but much less steeply, reaching about 22% at 240 e^-/s, or about 2 mM TMPD ("phase II").

It is immediately clear, once the interference of TMPD$^+$ is removed, that the steady-state reduction level of Fe_a in mitochondria is lower than previously thought [see, e.g., Wikström et al. (1976)]. What is less certain is exactly how much of the remaining absorbance change is actually attributable to reduction of Fe_a, at least in its normally functioning form. The multiphasic behavior of the data for the uncoupled state might be explained in terms of a subpopulation of inactive, or less active, cytochrome oxidase—perhaps enzyme in a pathway of biogenesis or biodegradation [see Haltia et al. (1989)]. In fact, the "phase I" phenomenon is what would be expected if the sample contained a subpopulation which behaved in the same way as the azide-inhibited mitochondria described below. Both in "phase I" and in the azide-inhibited mitochondria, at a given concentration of TMPD, the 605 minus 620 nm absorbance is higher in the uncoupled system than in the coupled system, consistent with the redox state of Fe_a being under thermodynamic rather than kinetic control (see below). If this is correct, it should be possible to arrive at the true reduction level of Fe_a by subtracting out the amplitude of "phase I". We have made this subtraction (for the uncoupled data only) when the Fe_a data are presented again in the summary Figure 5.

When high concentrations of TMPD were used, high con-

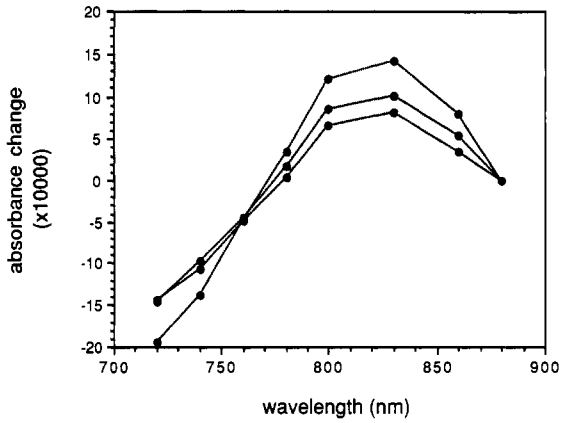

FIGURE 4: Steady-state minus reduced spectrum in the near-infrared at three different TMPD concentrations, showing the 830-nm Cu_A band: coupled mitochondria. TMPD concentrations (top to bottom): 5, 187, and 476 μM. Normalization at 880 nm is arbitrary. For these measurements, the reference wavelength was fixed at 830 nm, and the "measuring" wavelength was moved. Thus, the 830-nm point is not a measurement but is zero by definition. Cytochrome aa_3 concentration, 1.3 μM. Other conditions; see Materials and Methods.

centrations of FCCP were needed to produce the full effect on the steady-state redox levels of Fe_a (and Cu_A and Fe_c). Apparently, as the turnover rate is increased, it is possible to overwhelm the transport capacity of the FCCP [see Nicholls (1974)], especially given the large quantitites of mitochondria needed for spectroscopic work, and the high turnover rates which can be reached by using TMPD (see Materials and Methods).

It should be noted that the absorbance changes produced by adding FCCP to samples during turnover are not exactly the same as the Fe_a reduced minus oxidized spectrum. There is an increase in absorbance around 605 nm, but also an increase at about 580 nm which is not typical of Fe_a. Thus, the putative inactive subpopulation might have a slightly altered visible spectrum.

Measurement of the 830-nm Band in Intact Mitochondria. Oxidized Cu_A gives rise to a broad, weak absorption band centered around 830 nm (Beinert et al., 1980; Boelens & Wever, 1980). Measurement of this band in intact mitochondria is complicated by its width, together with the fact that mitochondrial suspensions scatter light significantly. Since this band is so broad, the measurement and reference wavelengths must be far apart, and this exacerbates the problems of light scattering.

The full reduced minus oxidized absorbance change in the near-IR could only be measured by averaging over a number of samples. It was somewhat easier to measure the reduced minus steady-state absorbance difference since this could be done by following the fast absorbance change which occurred when samples became anaerobic. In addition, this measurement could be repeated several times in the same sample by adding small volumes of hydrogen peroxide as a source of dioxygen (see Materials and Methods). The measurements were further complicated by the fact that the amplitude of absorbance changes at 830 nm was not a simple linear function of the concentration of mitochondria. This phenomenon, which is probably related to light scattering, meant that it was often impossible to make absolute comparisons of data from different batches of mitochondria, or from samples containing different amounts of mitochondria.

Figure 4 shows steady-state minus reduced (i.e., anaerobic) spectra in the near-infrared at three different TMPD concentrations. The spectra clearly show a band with the characteristic width of the 830-nm band of Cu_A. It is also clear

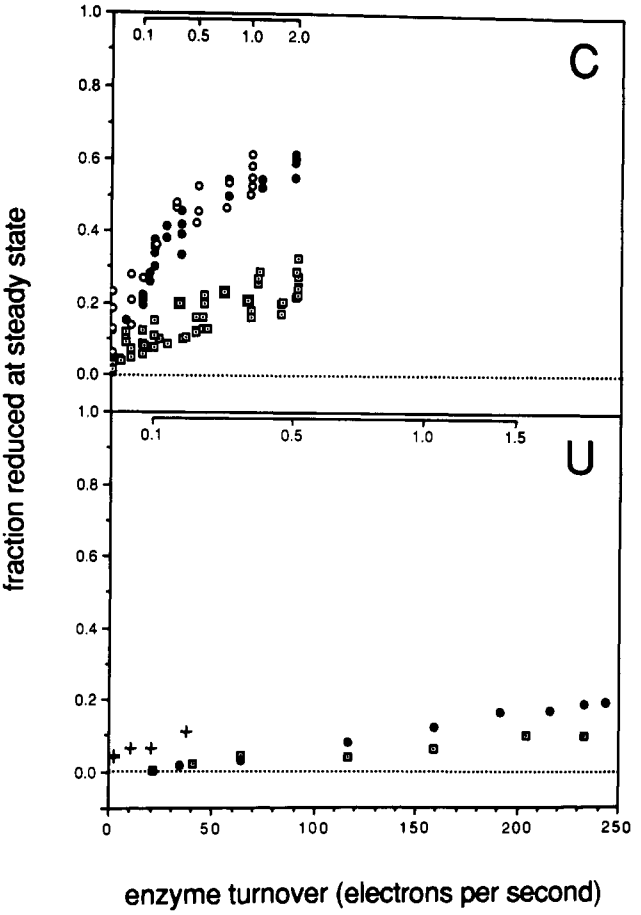

FIGURE 5: Fe_c, Cu_A, and Fe_a: steady-state reduction levels as a function of enzyme turnover. C, without FCCP; U, with FCCP; (●) Fe_c (550 minus 582 nm); (○) Cu_A (830 minus 880 nm); (□) Fe_a (605 minus 620 nm); (+) Fe_c (550 minus 540 nm; see text). The scale at the top of each frame gives TMPD concentration (millimolar). FCCP concentrations for spectroscopic measurements (panel U only): Fe_c (550 minus 582 nm), 3.5 μM; Fe_c (550 minus 540 nm), 0.2–0.4 μM; Fe_a, 3.5 μM. Fe_a data in panel U: the amplitude of "phase I" has been subtracted and the data rescaled (see text and Figure 3), and only points over 40 s^{-1} are shown.

that as the velocity of turnover is increased, the intensity of the band decreases, indicating that Cu_A is more reduced in the faster steady state.

These spectra were acquired point-by-point, by fixing the reference wavelength at 830 nm and moving the "measuring" wavelength. (The 830-nm point is thus zero by definition, and is not a measured value.) Each spectrum was measured in a single sample; the different points were obtained by means of repeated additions of hydrogen peroxide as an oxygen supply. As a check on our method, we made similar measurements using reference wavelengths at both ends of the 830-nm band (750 and 885 nm). All of these measurements gave substantially the same shape of curve. This supports the validity of the reduced minus steady-state measurement and argues against these results being artifacts produced by light-scattering changes and/or falloff in the sensitivity of the photomultiplier tube at longer wavelengths.

Steady-State Redox Behavior of Fe_c, Cu_A, and Fe_a. Figure 5 compares the levels of reduction of Fe_c, Cu_A, and Fe_a during steady-state turnover as a function of enzyme turnover rate. The data are combined from experiments with several different batches of mitochondria, and are given for the coupled (C) and uncoupled (U) systems in separate windows. The scale inset at the top of each window gives TMPD concentrations. Note that since the enzyme turnover scales for both windows are the same, the TMPD scales in the coupled and uncoupled

windows are necessarily different. Although there is considerable scatter in the coupled Fe_a data, much of this is due to differences between batches of mitochondria; the data from a single batch were more consistent than this, as indicated in Figure 3.

As described above, in order to avoid interference from $TMPD^+$, the reduction level of Fe_c was measured at 550 minus 580 nm, instead of the more usual 550 minus 540 nm. The data give the steady-state absorbance at these wavelengths as a fraction of the full reduced minus oxidized absorbance change without any compensation for the contribution from cytochrome c_1 (see Materials and Methods). When the uncoupled system was measured at 550 minus 580 nm, the first few points fell close to the base line, and the first increase in absorbance began at a nonzero TMPD concentration. This was apparently due to the absorbance increase at 580 nm associated with "phase I", described above. When these low TMPD points were measured at 550 minus 540 nm instead, the absorbance increased without a lag. We have included the first few points of the 550 minus 540 nm data in Figure 5. Since there should be very little $TMPD^+$ in these samples, they presumably indicate a more correct base line for the Fe_c data.

During coupled steady-state turnover, Fe_c can be driven to about 60% reduction, but the reduction level does not increase linearly with enzyme turnover. In the uncoupled system, at any given velocity, Fe_c is less reduced, and the dependence of reduction level on turnover rate appears to be linear.

Figure 5 (top) also shows the fraction of Cu_A reduced in the coupled steady state. As described above, these measurements were subject to a number of difficulties, and the data should be regarded as semiquantitative. In the coupled system, the redox behavior of Cu_A appears to follow that of Fe_c very closely, although there may be differences at very low turnover rates. The difference with respect to Fe_a is considerable by comparison.

In the uncoupled system, Cu_A appears to remain very oxidized indeed. Within the resolution of our measurements (about 10%), we were unable to measure any change in the reduced minus steady-state absorbance change either at 830 minus 880 nm or at 820 minus 750 nm, when TMPD concentration was varied over a wide range (the data are not shown). This indicates Cu_A is less than 10% reduced.

We also measured the reduction levels of Fe_c and Fe_a in coupled mitochondria respiring on succinate in order to be able to compare our results to physiological mitochondrial studies. When the comparably low rate of turnover with succinate is taken into account (about 5 and 50 e^-/s for the coupled and uncoupled states, respectively), there are no major differences in the levels of reduction of Fe_c and Fe_a in the two systems.

Are Fe_c, Cu_A, and Fe_a in Equilibrium during Turnover? If the electron transfer from the "low-potential" redox centers to the oxygen binding site is sufficiently rate-limiting, the metal centers which precede this step should be close to redox equilibrium during steady-state turnover. Several previous studies of this subject have worked from the assumption that this is the case (Wikström et al., 1976; Brunori et al, 1981). Applying this kind of analysis to our data, and assuming an E_m of 230 mV for cytochrome *c* bound to the mitochondria (Dutton et al., 1970; Erecinska et al., 1974), the apparent midpoint potential of Fe_a is approximately 195 mV for coupled turnover. In the uncoupled system too, Fe_a is consistently less reduced than Fe_c (assuming that the "phase I" intensity can be subtracted), giving an apparent E_m below 230 mV. This is significantly lower than the values (well above 300 mV)

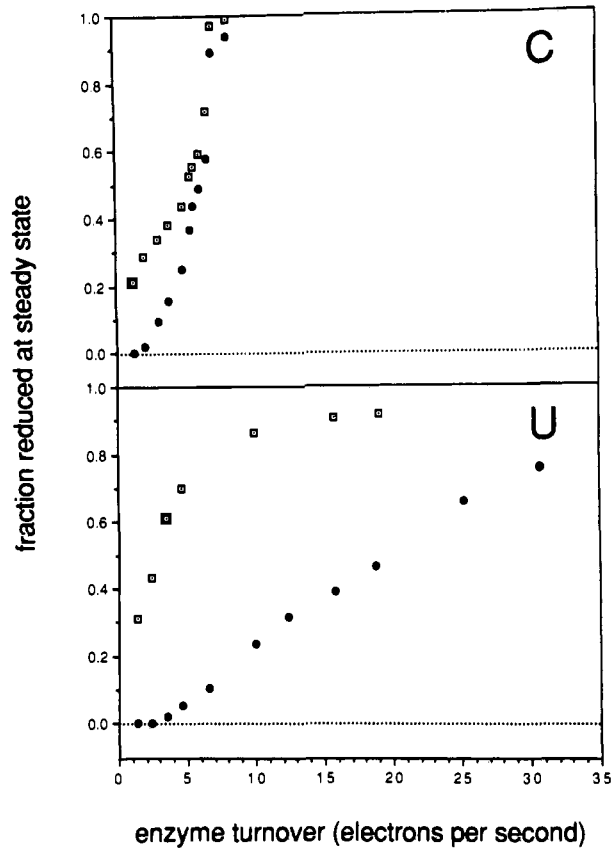

FIGURE 6: Azide-inhibited mitochondria: steady-state reduction levels of Fe_c and Fe_a as a function of enzyme turnover. C, without FCCP; U, with FCCP; (●) Fe_c; (⊡) Fe_a; azide concentration, 0.373 mM. Fe_a reduction was measured at 605 minus 620.5 nm; Fe_c reduction was measured at 550 minus 582.5 nm (see Materials and Methods). Note: The uninhibited activity of the mitochondria used in these activity measurements was only 80% (approximately) of the activity in Figure 1.

found in (nonturnover) redox titrations for the "upper asymptotic midpoint potential", which correspond to the situation in which both Fe_{a_3} and Cu_B are oxidized (Blair et al., 1986). These discrepancies led us to question the assumption of a near-redox equilibrium between Fe_a and Fe_c in these conditions (see Discussion).

One classical way to study the question of whether these centers are in equilibrium is to slow down the outflow of electrons from the system even further [see Muraoka and Slater (1969)]. If there is an equilibrium, then this should not alter the Nernstian relationship beween the redox levels, assuming that inhibition does not perturb the system in other ways. However, if the system is not at equilibrium to begin with, slowing down the rate of electron outflow may allow the system to approach equilibrium. If the resulting changes do not follow Nernst's law, it would suggest that the initial state was not an equilibrium. In order to study this possibility, we repeated the experiments in the presence of low concentrations of azide, which slows down the rate of turnover at the oxygen binding site.

In the presence of azide (Figure 6), both Fe_a and Fe_c became more reduced with increasing turnover, but the relationship between the reduction levels was altered dramatically with respect to the uninhibited case. With azide, the apparent E_m of Fe_a was now "raised" to about 245 mV during coupled turnover, and to about 310 mV during uncoupled turnover. These values are much more consistent with values reported in redox titrations (Wilson et al., 1972). Not only are the apparent E_m values in both coupled and uncoupled systems

higher but also in the presence of azide the difference between the coupled and uncoupled values (about 65 mV) is now much more consistent with the expected effect of the membrane potential on the redox poise of Fe_a vs Fe_c (Hinkle & Mitchell, 1970). It should be noted that there is significant enzyme turnover in the region where Fe_c appears to be completely oxidized. This may indicate that the measured zero point is slightly (5–10%) higher than the real fully oxidized value, perhaps due to a dilution artifact.

Another effect of azide on the redox behavior of Fe_a is also noteworthy. In the absence of the inhibitor or uncoupler (Figure 5), the extent of reduction of Fe_a appears to determine turnover velocity, which is consistent with the idea that Fe_a is the donor of electrons to the bimetallic center. However, in the presence of azide and FCCP, this is no longer the case. Fe_a appears to equilibrate with Fe_c but no longer behaves as the rate-controlling electron donor to the oxygen binding site (see Discussion).

When cyanide or formate was used instead of azide, the results were dramatically different. Although the level of Fe_a reduction was significantly higher compared to the uninhibited case, Fe_a reduction was much less sensitive to the concentration of TMPD. When azide is used, the system behaves as though, on the time scale of turnover, the effect of inhibition is averaged over the whole enzyme population. In contrast, with cyanide or formate, the system behaves as though, on the time scale of turnover, one subpopulation of the enzyme is completely inhibited, while another subpopulation is fully active, with the relative sizes of the subpopulations determined by the inhibitor concentration.

Discussion

This is the first time that Cu_A has been systematically studied during enzyme turnover in any system in which turnover can produce, and thus work against, an electrochemical proton gradient. We find that the redox state of Cu_A is sensitive to $\Delta\mu_H$. Under coupled conditions, Cu_A becomes significantly reduced during turnover, very much like Fe_c. This is consistent with the idea that in the coupled steady state these two centers are close to redox equilibrium with one another and that electron transfer between them is not affected by the $\Delta\mu_H$ [cf. Rich et al. (1988)]. Erecinska et al. (1974) cite steady-state E_h values for Cu_A which are in agreement with this conclusion. In the presence of uncoupler, however, Cu_A always remains highly oxidized.

Our reexamination of Fe_a in mitochondria revealed that it remains highly oxidized under all turnover conditions. We found that Fe_a is never more than about 30% reduced during steady-state turnover in the absence of terminal inhibitors. Even this value is achieved only in the coupled state, at extremely high turnovers that presumably never occur physiologically. In the presence of uncouplers, Fe_a is even less reduced. The redox behavior of Fe_a in the uncoupled system is distinctly biphasic, consistent with the presence of a less active subpopulation of the enzyme.

We also found that the accumulation of $TMPD^+$ can cause Fe_a reduction to be significantly overestimated. It appears that the high levels of steady-state reduction reported in the past are either artifacts due to the accumulation of $TMPD^+$ in the samples or results obtained with kinetically impaired forms of the enzyme (see below).

The original reports of $TMPD^+$ during steady-state turnover were made with submitochondrial particles (Sagi-Eisenberg & Gutman, 1979) and sonicated vesicles (Nicholls et al., 1988), in which all or part of the enzyme is in an inside-out orientation. The buildup of $TMPD^+$ was attributed to the activity of the inside-out enzyme with the idea that $TMPD^+$ produced inside the membrane boundary would be less accessible to externally added ascorbate. Later, Gregory and Ferguson-Miller (1989) observed the accumulation of $TMPD^+$ in a right-side-out system. Adapting an idea from Nicholls et al. (1988), they concluded that $TMPD^+$ produced on the outside of the vesicles was migrating to the inside electrophoretically before it could be re-reduced by ascorbate.

Neither of these mechanisms can explain our mitochondrial data. In mitochondria, there is no inside-out oxidase to generate $TMPD^+$ inside the mitochondrial matrix. Moreover, we have found that at a given TMPD concentration, samples which contain uncoupler accumulate more $TMPD^+$ than those without uncoupler. This result suggests that the membrane potential does not play an important role in the buildup of $TMPD^+$. It is therefore likely that the major reason for the $TMPD^+$ accumulation shown here during mitochondrial turnover is simply that the rate of oxidation of TMPD outstrips the rate at which ascorbate re-reduces $TMPD^+$.

If so, $TMPD^+$ might also be expected to accumulate when the detergent-solubilized enzyme is studied, provided that the enzyme preparation is capable of high turnover. High levels of steady-state Fe_a reduction have been reported in this system, but this apparently has to do with the slow electron-transfer kinetics in some isolated preparations and not $TMPD^+$. Mahapatro and Robinson (1990) have recently shown that in the detergent-solubilized enzyme, the rate of intramolecular electron transfer (Fe_a to Fe_{a_3}) is very sensitive to the detergent used. In detergents in which enzyme turnover is relatively slow, such as Triton X-100, the steady-state level of reduction of Fe_a was found to be much higher than it is in lauryl maltoside, a detergent which supports turnover rates as high as those in mitochondria. (Since they studied the oxidation of ferrocytochrome *c* by the oxidase, without ascorbate or TMPD, their numbers are not directly comparable to ours.) Unfortunately, lauryl maltoside was introduced relatively recently (Rosevear et al., 1980), and almost all of the steady-state redox studies to date have used the older, less favorable detergents.

Similarly, in the reconstituted enzyme, the steady-state redox level of Fe_a is sensitive to external factors. Gregory and Ferguson-Miller (1989) took samples of enzyme which had been isolated by a number of different procedures, reconstituted each one into vesicles, and studied the steady-state reduction levels of Fe_a, the sensitivity of these levels to uncoupling, and the RCR's. They found that preparations which exhibited low RCR's had high levels of steady-state reduction of Fe_a, and vice versa. After correction for $TMPD^+$ interference, they found that the preparations with the highest RCR values gave Fe_a reduction levels almost as low as those we have found in mitochondria [25–34%, as compared to 14–22% here, at the same TMPD concentration (0.3 mM)]. The preparations with the lowest RCR values had Fe_a reduction levels similar to those obtained with the enzyme dispersed in detergents which give low activity.

Thus, it appears that the high levels of steady-state reduction of Fe_a which have been reported in the past are artifactual. In some cases, this was the result of inhibition of the intramolecular electron-transfer rate either through harsh conditions during isolation (Gregory & Ferguson-Miller, 1989) or by dispersion in unfavorable detergents (Mahapatro & Robinson, 1990). In other cases, the high levels of reduction were artifacts of $TMPD^+$ (Gregory & Ferguson-Miller 1988a,b, 1989; Wikström et al., 1976). Although the reasons for these results were different, taken together they produced a consistent, but erroneous, picture of the steady-state behavior of Fe_a. It is

now becoming clear that Fe_a remains largely oxidized during steady-state turnover, not only in intact mitochondria but also in the more physiological of the isolated preparations. Futhermore, these results provide a more consistent picture of the steady-state behavior of the mitochondrial respiratory chain with different substrates. As described above, the reduction levels of Fe_c and Fe_a during turnover with ascorbate/TMPD are consistent with the levels observed during turnover with succinate [see Muraoka and Slater (1969)].

The behavior of the slower systems is similar to that of the azide-inhibited mitochondria. In both cases, during turnover, Fe_a appears to be approximately in equilibrium with Fe_c. For example, steady-state turnover experiments on the isolated enzyme in Tween 80 (where the turnover rate is about 3 e^-/s at 0.1 mM TMPD) indicate that in this system, Fe_a is (approximately) in equilibrium with Fe_c and operates at an E_m of 286 mV (resting) to 305 mV (pulsed; Brunori et al., 1979). These values, based on an E_m of 268 mV for soluble cytochrome *c* (Dutton et al., 1970), are close to the nonturnover potentiometric values. (Spectra acquired during steady-state turnover, albeit at only 30% of the maximum TMPD concentration, did not show the presence of $TMPD^+$, indicating that the high levels of reduction at Fe_a are real.) In azide-inhibited mitochondria (described above), the apparent E_m of Fe_a is about 245 mV during coupled turnover, and about 310 mV during uncoupled turnover, once again in good agreement with potentiometric values (Wikström, 1988b).

In native mitochondria (i.e., without inhibitors), these nonturnover redox relationships are no longer followed. In both the coupled and uncoupled systems, Fe_a remains more oxidized than predicted, indicating either that the prevailing midpoint potentials have changed or that the metal centers are not in equilibrium.

In the coupled system, Fe_a is always significantly less reduced than Fe_c and Cu_A. At the same time, the reduction levels at Fe_c and Cu_A do not increase as fast as enzyme turnover. Both of these features can be accounted for by assuming that the three centers are in redox equilibrium and that electron exit from Fe_a is (locally) rate-limiting. As described above, the steady-state redox data for the coupled system can be fitted reasonably well assuming that the E_m of Fe_a 35 mV lower than that of Fe_c. Given 230 mV for the latter (Dutton et al., 1970; Erecinska et al., 1974), this yields about 195 mV for Fe_a. (The inactive subpopulation, proposed to explain "phase I" of the uncoupled data, should not affect this analysis significantly, since the E_m of Fe_a would still be expected to be dependent on $\Delta\Psi$, in this subpopulation.) This is 50 mV lower than the value obtained with fully coupled, azide-inhibited mitochondria and more than 100 mV lower than the value for the uncoupled state. Even assuming that the system without azide can build up a larger $\Delta\Psi$, the Coulombic effect of the membrane potential (Hinkle & Mitchell, 1970) can still account for lowering of the E_m by no more than about 80 mV, assuming a membrane potential of 160 mV.

There is, however, an additional mechanism which might account for such a low E_m. There is an anticooperative redox interaction between the oxygen binding site and Fe_a; when Cu_B is reduced, the E_m of Fe_a is lowered by about 55 mV (Goodman, 1984; Rich et al., 1988; Wikström, 1988b). It is possible that Cu_B is largely reduced during coupled turnover, something which is worth investigating further. Alternatively, the oxygen binding site might assume a conformation in which this influence on Fe_a is manifest, without Fe_{a_3} or Cu_B is actually being reduced.

The alternative is that the E_m is not this low and that the redox level at Fe_a is controlled by kinetic constraints. This would lead to the conclusion that electron transfer from $Cu_A \rightarrow Fe_a$ and $Fe_c \rightarrow Fe_a$ (if it occurs at all) must be as slow as 200 s^{-1} (100 s^{-1}/0.5 occupancy). This would still not explain the fact that Fe_c and Cu_A fail to keep pace with enzyme turnover, and it would be necessary to invoke an alternative path for electrons which bypasses these metal centers. This seemingly unlikely possibility is discussed below, in connection with the biphasic kinetics.

In the uncoupled system, Fe_c, Cu_A, and Fe_a all remain very oxidized even at high turnover rates. Again, relative to Fe_c, Fe_a is less reduced than would be predicted from potentiometric data, but here it seems clear that these centers are not in redox equilibrium: Beginning from the coupled-state data (above), release of the $\Delta\Psi$ should raise the effective E_m of Fe_a from 195 mV up to 260 mV at the very least. On this basis, if there is an equilibrium, Fe_a should be at least 45% reduced when Fe_c reaches 20% reduction level. Even before the "phase I" absorbance contribution is subtracted (see above), it is clear that Fe_a is significantly more oxidized than this. Since the E_m of Cu_A is slightly higher than that of Fe_c (Erecinska et al., 1971; Rich et al., 1988), it also appears that these two centers are not in redox equilibrium. On the other hand, since Cu_A remains too oxidized to follow, it is not possible to answer the more interesting question of whether Fe_a and Cu_A are in redox equilibrium during steady-state turnover.

The low levels of reduction in the uncoupled system indicate that the rate constants for electron transfer within the oxidase must be very high. Given that Cu_A is less than 10% reduced in the uncoupled system, if all the electrons flow sequentially through Cu_A and Fe_a, the (time-averaged) rate constant for the $Cu_A \rightarrow Fe_a$ electron transfer would have to be at least 2500 s^{-1} in order to sustain a flow rate of 250 s^{-1} (the maximum observed). The rate constants for electron transfer from Fe_a and/or Cu_A to the oxygen binding site would also have to be at least this large. This is faster than the slowest phase (600 s^{-1}) observed when fully reduced cytochrome oxidase is reoxidized in the flow–flash experiment (Hill et al., 1986; Oliveberg et al., 1989). It is also not a great deal slower than rates of 6000–10 000 s^{-1} which have been measured for other internal electron transfers in the enzyme (Hill et al., 1986; Kobayashi et al., 1989; Morgan et al., 1989; Oliveberg et al., 1989).

Both the coupled and the uncoupled systems display biphasic kinetics with respect to TMPD concentration. Similar behavior is observed when cytochrome *c* oxidase turnover is studied at different cytochrome *c* concentrations. Biphasicity in that system is thought to arise either because the oxidase has two different binding affinities for cytochrome *c* or because the properties of the oxidase itself (in the time average) change as it is turned over more quickly [see review by Cooper (1990)]. The biphasicity observed here is unlikely to arise from two different affinities of cytochrome oxidase for cytochrome *c*: First, in mitochondria, the total amount of cytochrome *c* is constant; and second, even though the concentration of *ferro*cytochrome *c* does change, the reduction level at Fe_c in the coupled system does not keep pace with the rate of turnover. (If our data are replotted, in a Eadie–Hofstee plot, considering Fe_c^{2+} as "substrate", V/S in the high-activity phase of the coupled system no longer decreases, but increases with increasing enzyme turnover.)

It is possible that the high-activity kinetic phase arises from electrons bypassing Fe_c, Cu_A, and perhaps also Fe_a. However, this also appears unlikely for several reasons: (a) Fe_c appears

to be an essential part of the electron pathway; high turnover rates are not observed in mitoplasts depleted of cytochrome *c*. (b) In the uncoupled system, the reduction levels at Fe_c and Fe_a continue to increase in proportion to turnover well beyond the point where a low-activity phase would be expected to saturate, (c) the same is true of Fe_a in the coupled system. It is possible that cytochrome *c* manifests its influence "allosterically" and that it is not always necessary for it to be part of the electron pathway, but all of this taken together indicates that the biphasic behavior arises either from low- and high-activity interactions of TMPD itself or from something in the enzyme's internal mechanism. More precise data will be needed for a complete analysis of these phenomena.

It appears that the supply of electrons from TMPD is always ultimately rate-limiting. In the uncoupled system, it is clear that none of the metal centers would be anywhere close to full reduction at the extrapolated V_{max}. The coupled system is more complicated. Here too, Fe_a would be far from fully reduced at V_{max}. A linear extrapolation of the redox level of Fe_c (or Cu_A) would reach 100% at about V_{max}, but it is not clear what physical significance this would have, since neither center behaves in a linear way up to this point.

To summarize, in azide-inhibited mitochondria, the reaction at the oxygen binding site is rate-limiting. Fe_a and Fe_c are approximately in redox equilibrium in accordance with the known dependence of E_m on $\Delta\Psi$. (The redox behavior of Cu_A is still being studied.) In coupled mitochondria without terminal inhibitors, turnover is faster, and the exit of electrons from Fe_a is locally rate-controlling. Fe_c and Cu_A appear to be in redox equilibrium. However, if Fe_a is in redox equilibrium with these two centers, its E_m must be 20–50 mV lower than can be accounted for from the dependence of the E_m on $\Delta\Psi$. One possible explanation for this is the anticooperative redox interaction between Cu_B and Fe_a, provided that Cu_B is reduced during turnover. The alternative, that the E_m is not this low, leads to the conclusion that Fe_a is not in equilibrium with Fe_c and Cu_A and that electron transfer from $Cu_A \rightarrow Fe_a$ and $Fe_c \rightarrow Fe_a$ (if it occurs at all) must be as slow as 200 s^{-1} (in the time average). When $\Delta\mu_H$ is dissipated by adding uncoupler, Fe_c, Cu_A, and Fe_a all become more oxidized. The E_m of Fe_a increases, as does the rate constant for electron exit from this center. This clearly indicates that there is respiratory control exerted "beyond" Fe_a. As a result of these changes, neither Fe_a nor Cu_A is now in redox equilibrium with Fe_c. (Our data cannot answer the question of whether Cu_A is in equilibrium with Fe_a under these circumstances.)

Finally, our results provide evidence of two electron-transfer pathways through the enzyme. As noted above, in the absence of both FCCP and azide, the reduction level of Fe_a is an almost linear function of the turnover rate while that of Fe_c is not. This suggests that electrons can flow directly from Fe_a to the oxygen binding site and that the reduction level of Fe_a may have a primary role in determining the rate of enzyme turnover. This situation changes dramatically in the presence of azide. Here it seems as if flux between Fe_a and the oxygen binding site has been strongly inhibited and that (at least in the uncoupled system) the rate of turnover is primarily controlled by Fe_c (or more probably Cu_A). This suggests that, at least under some circumstances, Cu_A can donate electrons directly to the oxygen binding site. Whether both of these pathways are operating simultaneously in the uninhibited enzyme is not known. If there are two active pathways, the proton pump might be coupled to only one of the them (Blair et al., 1985). This is an interesting possibility since it has recently been demonstrated that the four electron transfers required to reduce O_2 at the bimetallic center are not equivalently linked to proton translocation (Wikström, 1989).

Acknowledgments

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Registry No. Cu, 7440-50-8; Fe, 7439-89-6; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6; cytochrome *a*, 9035-34-1.

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Effect of Actin, ATP, Phosphates, and pH on Vanadate-Induced Photocleavage of Myosin Subfragment 1†

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ABSTRACT: Near-UV irradiation in the presence of vanadate cleaves the heavy chain of myosin subfragment 1 at three specific sites located at 23, 31, and 74 kDa from the N-terminus. Increasing the pH from 6.0 to 8.5, gradually, reduces the efficiency of the cleavage and comletely eliminates the 31-kDa cut. Actin specifically inhibits the photocleavage at the sites located 31 and 74 kDa from the N-terminus. ATP strongly protects from cleavage at the 23- and 31-kDa sites and less strongly from the cut at the 74-kDa site. ADP and pyrophosphate have similar, but less pronounced, effects as ATP. Orthophosphate inhibits the photocleavage at the 23- and 74-kDa sites with a similar efficiency. In the ternary actin-S-1-ATP complex, the photocleavage is inhibited at all sites, and the effects of actin and ATP are additive. Photocleavages affect the K^+(EDTA)-, Ca^{2+}-, and actin-activated ATPase activity of subfragment 1. Loss of all three ATPases is caused by cleavage at the 23-kDa site, while the cut at the 74-kDa site only leads to the loss of actin-activated ATPase activity. It is concluded that subfragment 1 contains at least two distinct phosphate binding sites, the first being part of the "consensus" ATP binding site wherein the 23-kDa photocleavage site is located. This site is responsible for the binding and hydrolysis of ATP. It is possible that the 31-kDa cleavage site is also associated with the "consensus" site through a loop. The 74-kDa cleavage site is a part of another phosphate binding site which may play a role in the regulation of the myosin-actin interaction.

Myosin is a ubiquitous protein in eukaryotes, responsible for biological motility and muscle contraction by coupling the actin interaction with the hydrolysis of ATP. Distinct ATP and actin binding sites reside on the head segment of myosin, named subfragment 1 (S-1).[1] Because of the biological significance of the myosin-ATP and myosin-actin interactions, it is essential to characterize the respective binding sites of ATP and actin on S-1. The finding that the S-1 heavy chain can be cleaved into three trypsin-resistant fragments [27, 50, and 20 kDa, aligned in this order from the N-terminus (Balint et al., 1978; Mornet et al., 1979)] provided a convenient framework for the localization of the binding sites.

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[1] Abbreviations: S-1, subfragment 1 of myosin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTE, dithioerythritol; IAA, iodoacetamide; HC, heavy chain; LC, light chain; LB, line-broadening parameter for the exponential multiplication of free induction decay (FID).

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