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Reaction of Formate with the Fast Form of Cytochrome Oxidase: A Model for the Fast to Slow Conversion†

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ABSTRACT: The ability to isolate preparations of cytochrome oxidase which are highly homogeneous has facilitated a study of the effects of various reagents on the purified enzyme. The addition of either sodium formate, formamide, formaldehyde, or sodium nitrite to enzyme which reacts in a single rapid kinetic phase with cyanide causes a blue-shift of 4-6 nm of the net (cytochrome a + cytochrome a_3) Soret maximum. Only the derivative prepared by adding sodium formate demonstrates measurable intensity in the $g' = 12$ region of the low-temperature electron paramagnetic resonance (EPR) spectrum. This $g' = 12$ resonance is characteristic of cytochrome oxidase which has undergone a modification at the binuclear center and thereby reacts sluggishly with cyanide. As the site of cyanide binding in resting enzyme as been demonstrated to be Cu_B [Yoshikawa, S., & Caughey, W. S. (1990) *J. Biol. Chem. 265*, 7945-7958], it is proposed that formate can bind to Cu_B and the fast to slow transition is rationalized by using this proposal. The $g' = 12$ signal is also produced upon the addition of sodium formate to mitochondrial preparations, suggesting that the species responsible for this behavior may have possible physiological relevance. Physical properties of the formate derivative and data for other reagents reacted with the fast-reacting enzyme preparation are presented.

Preparations of cytochrome oxidase have been shown to exhibit variable spectral and kinetic properties (Baker et al., 1987; Palmer et al., 1988), and the fact that purified preparations of the enzyme are inhomogeneous is now generally accepted.

Early studies by van Buuren (1972) and Kumar et al. (1984) illustrated the problem of heterogeneity by showing multiple kinetic phases during the binding of cyanide to the purified enzyme. Kumar et al. further demonstrated that the phases of the reaction varied from preparation to preparation. Baker et al. (1987) then discovered that most purified samples consist

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mainly of two forms of the enzyme. The form which reacts rapidly with cyanide is referred to as fast enzyme and is equated with pulsed enzyme. The slowly reacting species is dominated by a much slower phase in its reaction with cyanide and is found to be responsible for the $g' = 12$ resonance in the low-temperature electron paramagnetic resonance (EPR)[1] spectrum. This $g' = 12$ signal is not present in mitochondrial preparations used as the starting point of the Hartzell–Beinert protocol and appears only late in the purification process. The basis for this heterogeneity has been a recent concern for our laboratory and has led to small modifications of the original Hartzell–Beinert procedure which result in an enzyme preparation which reacts rapidly and homogeneously with cyanide (Baker et al., 1987).

Fast enzyme[2] is characterized by a net observed Soret maximum of 424 nm at pH 7.9–8.1 and a single fast kinetic phase (k_{obs} = 0.04–0.08 s^{-1} in the pseudo-first-order reaction with saturating levels of cyanide. This enzyme does not possess the $g' = 12$ signal seen in the EPR spectra of many enzyme preparations. A homogeneous sample of the slow-reacting species can be prepared by incubation of fast enzyme at low pH and low enzyme concentration for several hours. Slow enzyme exhibits a Soret maximum at 417 nm at pH 6.8 and a single slow kinetic phase for its reaction with cyanide (k_{obs} = 0.0001–0.0002 s^{-1}). Moreover, the presence of this slow phase has been correlated with the presence of the $g' = 12$ signal (Baker et al., 1987), with the amplitude of the $g' = 12$ signal being proportional to the amount of enzyme which reacts slowly with cyanide. In the conversion of the fast enzyme to the slow form, the Soret MCD spectrum is unchanged, implying that the absorbance changes are due to cytochrome a_3 rather than cytochrome a since a change in the cytochrome a absorption maximum would result in a corresponding change in the zero-crossing at 426 nm in the MCD C term.

In an effort to define the structural consequences of the fast to slow conversion, we previously compared resonance Raman (RR) characteristics of these two species (Schoonover et al., 1988). The results suggested a global conformational change which affects both the cytochrome a and the cytochrome a_3 sites. In the course of that study, we discovered that the species formed upon reaction of our fast preparation with millimolar levels of sodium formate altered the RR spectrum to that observed with slow enzyme (Schoonover et al., 1988). Furthermore, sodium formate addition induces a blue-shift of the net Soret position which is independent of pH. The present study was undertaken to characterize the effects of formate and to determine which other reagents, if any, produce similar changes.

Materials and Methods

Solubilized bovine heart cytochrome oxidase was prepared as previously described (Baker et al., 1987). The final precipitate was dissolved in 50 mM HEPES containing 0.10–0.15% dodecyl maltoside at concentrations greater than 400 μM in cytochrome oxidase (i.e., >800 μM in heme a). This buffer system was used for studies at pH 6.8–8.2, while 100 mM phosphate buffer containing 0.10–0.15% dodecyl maltoside was used when pH values between 6.0 and 6.8 were used. Enzyme concentrations were calculated in terms of heme a by using the difference (reduced minus oxidized) extinction coefficient of 24 mM^{-1} cm^{-1} at 605 nm. The absence of heterogeneity in the purified enzyme was routinely verified by cyanide kinetics experiments and by EPR spectra to check for the absence of intensity in the $g' = 12$ region. The cyanide kinetics experiments used high, saturating levels of cyanide so that the reported rates are in the form of the limiting, pseudo-first-order rate constant. The sample of the Volpe–Caughey preparation used for EPR was supplied by Dr. O. Einarsdottir (Los Alamos National Laboratory).

Sodium formate (Mallinckrodt) was dissolved in water to make stock solutions of 1–4 M with the pH adjusted to 8.0–8.1. Sodium nitrite (Aldrich) stock solutions were made in the same manner. Formamide (Aldrich) and 37% formaldehyde solutions (Mallinckrodt) were diluted in buffer for stock solutions of 1–4 M. Likewise, stock solutions of the various anions studied were made to 1 M at pH 8.0–8.1. In the absorption and MCD experiments, the appropriate amount of stock reagent was added to 5–10 μM enzyme to give a final reagent concentration of 10–100 mM. In the EPR experiments, 50 μM enzyme and 50–100 mM reagent were used. The mixed-valence formate derivative was prepared by the addition of 100 mM sodium formate to fast enzyme followed by the addition of TMPD and sodium ascorbate to final concentrations of 0.5 and 3 mM, respectively. In the rapid-freeze EPR studies, the appropriate amounts of sodium formate and enzyme were placed in separate syringes and simultaneously added to a mixing chamber which emptied into an EPR tube. The resulting solution was frozen in a bath consisting of 2-methylbutane/methylcyclohexane (4:1) and dry ice. Samples were frozen in time intervals ranging from 30 s to 2 h.

The kinetics of cyanide binding were followed optically at 428 nm, while the reaction with sodium formate was monitored at either 411 or 418 nm. In both experiments, the reaction was initiated by addition of 50 μL of the appropriate reagent to 1.95 mL of 5 μM enzyme (aa_3) using an adder mixer (Precision Cell). The reaction was performed at ca. 15 °C on a computerized Cary 17 UV–visible spectrophotometer. Kinetic data were transferred from the Cary 17 to a Macintosh II (Apple Computer) and manipulated by using Cricket Graph (Cricket Software) or StatView II (Abacus Concepts).

Sodium [^{14}C]formate (Amersham Corp.) was utilized to check for the removal of bound formate. A solution of sodium formate with ^{14}C-radiolabeled sodium formate at pH 8.0 was reacted with fast enzyme. This enzyme solution was passed through a 1.5 × 11.0 cm Bio-Gel P6 (Bio-Rad) column developed with the HEPES/dodecyl maltoside buffer. Aliquots were collected off the column, the absorbance at 418 nm was monitored and the sample was then dispersed in scintillation fluid. The resulting solution was counted by using a Beckman LS 3801 liquid scintillation system. The scintillation fluid consisted of a toluene/ethanol solution with PPO as the primary fluor and POPOP as the secondary fluor.

The pH values were measured at room temperature using a Radiometer Model PHM63 pH meter. The optical spectra were obtained either on an IBM 9430 or on a Cary 17 UV–visible spectrophotometer controlled by a Compaq Deskpro 286 computer. EPR spectra were recorded between 10 and 20 K on a Varian E6 spectrometer; the instrumental conditions were the following: modulation amplitude, 20 G; microwave power, 3 mW; microwave frequency 9.2 GHz. MCD spectra were obtained with a Jasco 500C spectrometer using a 1.3-T

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ADP and ATP, adenosine di- and triphosphate, respectively; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; MCD, magnetic circular dichroism; RR, resonance Raman; EPR, electron paramagnetic resonance.

[2] For brevity, we will refer to the forms of cytochrome oxidase which react in a single fast or single slow kinetic phase with cyanide as fast and slow enzyme, respectively.

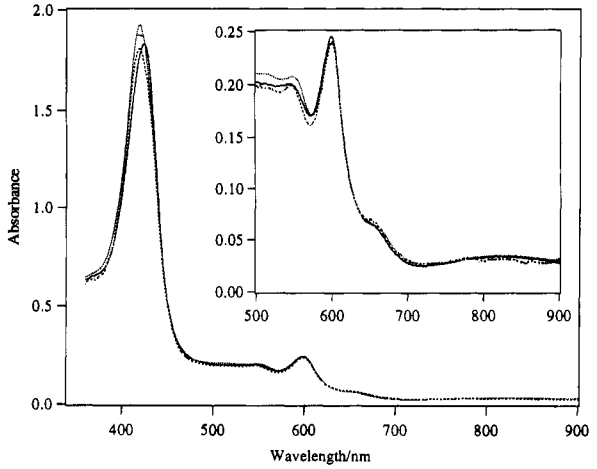

FIGURE 1: Effect of various reagents on the optical spectrum of the fast form of cytochrome oxidase. The spectra of fast enzyme (—), fast enzyme with 100 mM sodium formate (···), and fast enzyme with 100 mM formamide (- - -) were recorded at 20 μM in heme *a* and pH 8.1 in a 1-cm path-length cell. The inset shows the visible and near-IR regions on an expanded scale.

electromagnet. The $g' = 12$ species was quantitated in arbitrary units by expressing its peak-to-trough amplitude as a fraction of the amplitude of the $g = 3$ signal due to cytochrome a^{3+} and using the same signals measured in a sample of homogeneous slow enzyme as a reference. It should be noted that the absolute quantitation of non-Kramer's states such as that represented by the $g' = 12$ signal is a very specialized procedure [see Hendrich and Debrunner (1989)].

RESULTS

Absorption Spectra. The changes in the optical spectrum observed upon addition of sodium formate to fast enzyme at pH 8.1 are illustrated in Figure 1. The most striking feature is the blue-shift of the Soret band from 424 to 418–419 nm accompanied by a small increase in intensity. The changes in the visible region are more subtle and consist primarily of a slight red-shift of the α band—most apparent as a loss of intensity on the high-energy side of this band, a red-shift of the 655-nm band to ca. 665 nm, and increases in intensity near 516 and 560 nm (Figure 1, inset). No change was detected in the 830-nm band. These optical changes are much the same as those previously reported by Nicholls (1975, 1976).

A similar shift of the Soret band to lower wavelength is also observed upon the addition of 100 mM formamide to the enzyme, with the Soret maximum again changing from 424 to 418–419 nm at pH 8.1. The α band exhibits a red-shift similar to that found with the formate derivative though to a lesser extent, while there are only very slight changes in intensity between 450 and 580 nm. The one distinct difference between the effects of the two reagents is found in the 655-nm band. As noted above, this feature shifts to 665 nm upon addition of sodium formate while adding formamide only produces an increase in the intensity of the band at 655 nm.

Formaldehyde at relative low concentrations (<75 mM) shifts the Soret in a similar manner, while higher concentrations of this reagent (>100 mM) produce extensive denaturation of the enzyme; indeed, some denaturation occurs at all concentrations of formaldehyde as gauged by the appearance of turbidity upon addition of the aldehyde.

Addition of sodium nitrite, a species which is isoelectronic with sodium formate, shifts the Soret maximum from 424 nm to about 420 nm (data not shown). There is some uncertainty in this value, however, because of overlap with the intense absorption band present at 355 nm which is due to sodium nitrite itself. The spectral changes in the visible region are similar to those observed when sodium formate is added to fast enzyme except that they are more pronounced; the increase in intensity near 516 and 560 nm is more apparent as is the change in the α band. Furthermore, in the presence of nitrite, the 655-nm band shifts to ca. 675 nm rather than 665 nm found in the formate derivative. Some of these optical changes have been observed previously using difference spectroscopy (Paitain et al., 1985).

By contrast, sodium chlorite does not produce effects similar to those found with sodium nitrite and sodium formate. Although millimolar concentrations of sodium chlorite cause a small red-shift of the Soret maximum (1–2 nm) together with a loss of intensity in the α band, the small red-shift of the Soret is probably caused by the high pH of freshly prepared sodium chlorite solutions (lowering the pH causes an increase in chlorous acid which quickly decomposes to chlorine dioxide), while the loss in intensity may well be due to the powerful oxidizing capability of the chlorite solution.

In an attempt to find the other compounds which behaved similarly to sodium formate, various reagents were added to rapid enzyme. Sodium acetate, sodium trifluoroacetate, and glycine, which are structurally related to formate, do not significantly affect the optical spectrum of fast enzyme when used at the same concentrations. Methyl formate and ethyl formate do cause a slow shift of the Soret maximum to 418 nm, but as both esters hydrolyze readily to formate and the appropriate alcohol, is seems likely that formate is the reactive species in both cases.

In contrast to cyanide, which reacts rapidly with fast enzyme, shifting the Soret maximum to 428 nm (Baker et al., 1989), neither sodium cyanate nor sodium thiocyanate produces any immediate effect on the optical spectrum of fast enzyme. Over a period of several hours, sodium cyanate does shift of the Soret maximum to 428 nm, but this shift appears to be due to residual cyanide present in this reagent.

Among other reagents tested, 100 mM sodium azide shifts the Soret maximum of fast enzyme from 424 to 425–426 nm and also red-shifts the α and 655-nm bands, while sodium fluoride at the same concentration blue-shifts the Soret maximum at 423 nm and 655-nm band to a slightly lower wavelength. Hydrogen peroxide producers a rapid shift from 424 to 428 nm along with an increase in intensity of the α band.

Recent studies in other laboratories (Antonini et al., 1988a,b; Reimann et al., 1988; Kadenback et al., 1988) have described small spectral perturbations induced by adenosine triphosphate (ATP) and other anions. We have examined the effect of a series of anions including chloride, bromide, nitrate, carbonate, sulfate, perchlorate, phosphate, and ATP on the optical properties of our preparation of cytochrome oxidase. In each case, a slight red-shift (between 0.4 and 1.0 nm) of the net Soret maximum is noted upon the addition of these anions to oxidized enzyme; because of the small magnitude, this shift is best observed using difference spectroscopy. Small increases in intensity at 604 nm and between 500 and 590 nm appear to be substantially due to a small amount of reduction accompanying the addition of anions because this intensity is lost upon the addition of ferricyanide. It is important to note that these general anion-induced changes in the Soret maximum are in the opposite direction and much smaller that the 4–6-nm blue shift observed with sodium formate, formamide, formaldehyde, and sodium nitrite.

A summary of the effect of reagents examined on selected spectral parameters is presented in Table I.

Table I: Effect of Various Reagents on the Fast Form of Cytochrome Oxidase[a]

sample reagent	Soret (nm)	$g' = 12$ signal	k_{obs}(CN) (s^{-1})
fast enzyme	424.0		0.04–0.08
slow enzyme (pH 6.7)	417.0	yes	0.0001–0.0002
HCOONa	418.5	yes	0.0001
HCOONa (pH 6.2)	416.0	yes	0.0001
$H_3CCOONa$	424.0		
$F_3CCOONa$	424.0		
$HOOCCH_2NH_2$	424.0		
H_2CO	418.5		
$HCONH_2$	418.5		0.001–0.002
$NaNO_2$	420.0		0.001
NaCN	428.0		
NaF	422.2		
NaN_3	425.0		
H_2O_2	428.0		
$NaHCO_3$	425.0		

[a] pH 8.0–8.1 in all cases unless otherwise indicated.

MCD Spectra. The Soret MCD spectra of fast enzyme and fast enzyme reacted with sodium formate are very similar (Figure 2A). The zero-crossing at 426 nm is unchanged with slight intensity changes at 450 nm and between 410 and 420 nm being the only noticeable differences. The observed shift in the Soret of the absorption spectrum must then be due to cytochrome a_3 since the absence of any change in the MCD zero-crossing implies that the location of the absorption maximum of cytochrome *a* in unaffected. Essentially identical MCD spectra were obtained with formamide and sodium nitrite.

In contrast to the Soret region, the visible MCD spectra of these derivatives are clearly different despite the strong similarity in the visible absorption spectra. The spectrum of the formate derivative shows two distinct changes (Figure 2B). First, there is a significant decrease in MCD intensity beween 520 and 550 nm resulting in the appearance of a small feature at 555 nm. Second, the derivative-like feature centered at 662 nm in the unreacted enzyme shifts to about 670 nm upon addition of sodium formate. This change is reflected in the absorption spectrum as a shift in the 655-nm band to 665 nm. With formamide, a loss of intensity is again observed between 520 and 550 nm but to a lesser extent than noted with formate. As a result, the feature at 555 nm is more prominent in the formamide MCD spectrum. Furthermore, no shift of the derivative feature near 662 nm is observed, though there is an overall increase in MCD intensity between 625 and 700 nm which results in better resolution of the 655-nm band in the absorption spectrum. The addition of sodium nitrite (data not shown) results in a greater loss of MCD intensity from 520 to 570 nm, and only a very slight shoulder is evident near 555 nm. The derivative feature at about 670 nm is both red-shifted and significantly broadened with the result that the 655-nm band seen in the absorption spectrum of untreated enzyme is shifted to 675 nm.

The optical and MCD changes obtained on addition of formate are similar to those noted when fast enzyme is incubated at low pH to produce the slow form of the enzyme (Baker et al., 1987). In the experiments with slow enzyme, stock enzyme is diluted in pH 6.2 buffer to 10–20 μM for 48–72 h. This treatment shifts the Soret maximum from 424 nm to between 416 and 417 nm and leads to both optical and MCD spectra similar to those produced by addition of sodium formate (Baker et al., 1987; Palmer et al., 1988). To be certain that the changes induced by the addition of sodium formate are independent of pH, the pH of all reagents was adjusted to 8.0–8.1, and enzyme solutions were buffered at pH 8.1. At

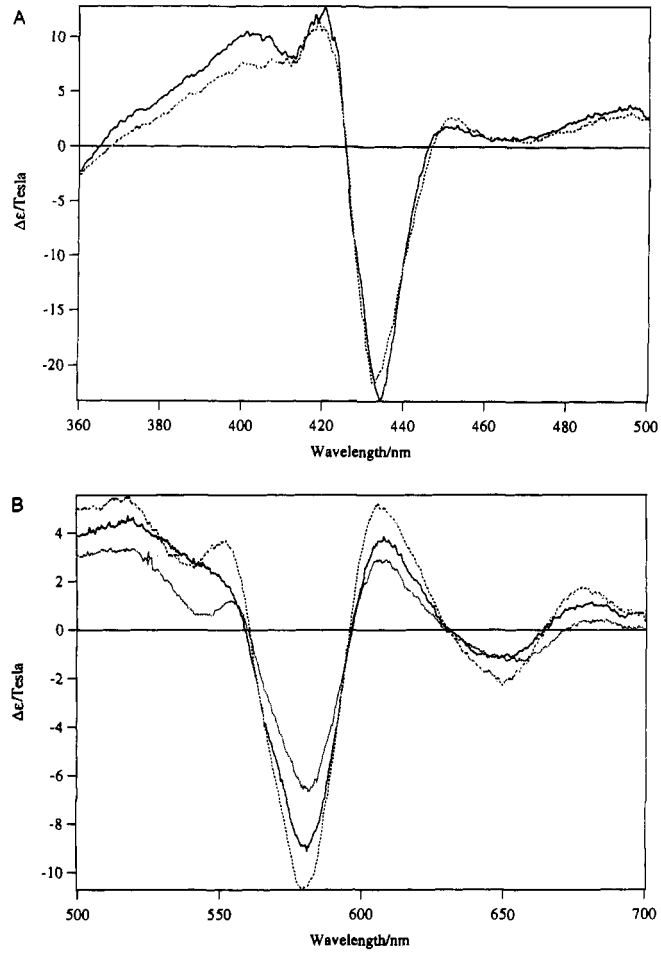

FIGURE 2: Comparison of the MCD spectra of the fast form of cytochrome oxidase reacted with different reagents in the Soret and visible regions. (A) Comparison of fast enzyme (—) and fast enzyme + formate (···). (B) Comparison of fast enzyme (—), fast enzyme + formate (···), and fast enzyme + formamide (- - -). The samples are those described in Figure 1. Soret spectra are the average of two scans using a fixed spectral bandwidth of 2 nm and a time constant of 1 s. Visible spectra were also the average of two scans with the same time constant using a fixed slit width of 180 μm; this resulted in a spectral bandwidth which increased from 3.6 to 10 nm over the range 500–700 nm. All spectra were smoothed by a single pass through a Gaussian filter.

this pH, our preparations of cytochrome oxidase do not interconvert to the slow form during the time used for these experiments as indicated by the lack of change in the position of the Soret maximum and by the lack of formation of the $g' = 12$ EPR signal. Longer incubations (several hours) did lead to a small blue shift of the Soret and the formation of small amounts of EPR signal at $g' = 12$.

pH Effects. The position of the Soret maximum of enzyme which has not been exposed to formate is known to show a substantial dependence on pH (Baker et al., 1987; Fabian & Malmstrom, 1989). This pH dependence is almost eliminated by the presence of formate.

Thus, changes in pH affect the wavelength of the Soret maximum of both fast and slow enzyme preparations. The Soret maximum of slow enzyme prepared by incubation at pH 6.2–6.8 exhibits a Soret band between 416 and 418 nm. Addition of formate does not lower this value further. Raising the pH to 8.5 shifts the Soret maximum of this slow enzyme to 423 nm compared to 425 nm for the fast enzyme sample at this pH. By contrast, exchanging the formate species prepared at pH 6.2 into formate-free buffer, pH 8.5, hardly affects the position of the Soret maximum.

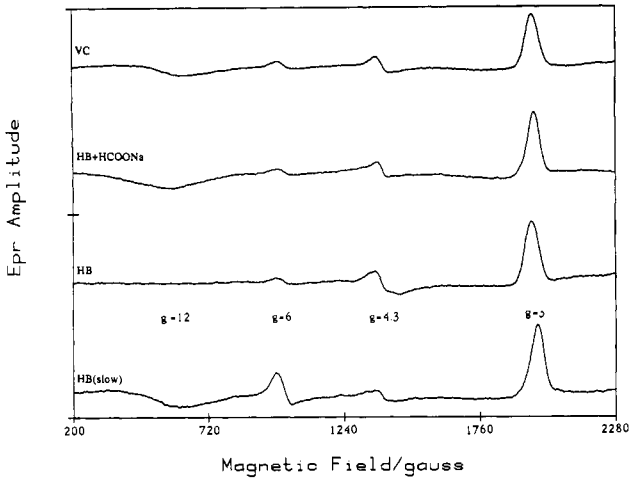

FIGURE 3: Low-field EPR spectra of various preparations of cytochrome oxidase. VC, Volpe–Caughey preparation; HB+HCOONa, fast enzyme from the modified Hartzell–Beinert preparation in the presence of 100 mM sodium formate; HB, fast enzyme from the modified Hartzell–Beinert preparation; HB (slow), enzyme from the HB preparation incubated at 10 μM (heme *a*) at pH 6.2 for 72 h followed by concentration. All sample concentrations were approximately 100 μM in heme *a*.

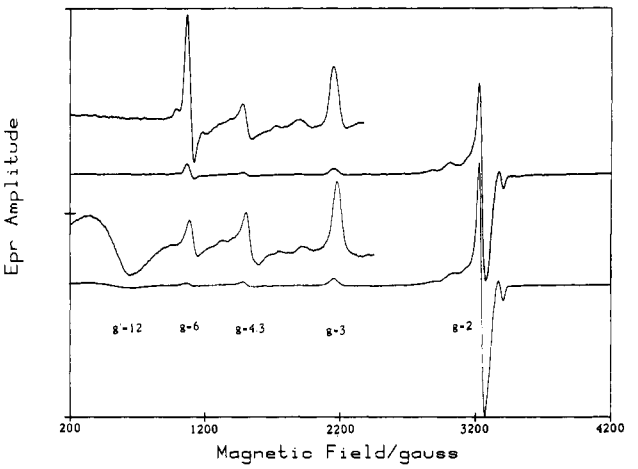

FIGURE 4: Comparison of EPR spectra of mitochondrial preparations as isolated and in the presence of 50 mM sodium formate. The mitochondrial preparation (top trace) is from the starting point of the modified Hartzell–Beinert preparation while an aliquot of the same sample was exposed to 50 mM sodium formate (bottom trace). Both samples were incubated on ice for ca. 45 min before being frozen for EPR spectra. The low-field region of each is expanded by a factor of 10 to better visualize the appearance of the $g' = 12$ signal.

Fast enzyme preparations also demonstrate a variation in the net Soret maximum with changes in pH, with the position of the Soret maximum increasing with increasing pH. As isolated, fast enzyme possesses a Soret maximum at 424 nm, and at pH 8.0–8.1, this Soret position is stable for several hours under our experimental conditions. Raising the pH to 8.5 slightly shifts the Soret to about 425 nm; the maximum value observed with increasing pH is 426 nm at pH 9.5 (Baker & Palmer, 1987). Between pH 6.2 and 7.6, the Soret maximum shifts to lower wavelengths, but this is a complex process because, under these conditions, fast enzyme spontaneously converts to the slow form. Thus, upon dilution of fast enzyme in buffer at pH 7.7, there is an immediate shift of the Soret maximum from 424 to 422.5 nm, while enzyme diluted into pH 6.8 buffer exhibits a value of 422 nm. Subsequent prolonged incubation at this latter pH (24–75 h) leads to a further shift to 418–419 nm. By contrast, the addition of sodium formate to fast enzyme at pH 8.1 shifts the Soret band from 424 to 418–419 nm; this change is complete in 1 h. Subsequently, lowering the pH to 6.2 immediately shifts the band further, to about 416–417 nm.

EPR Spectra. The low-temperature (15 K), low-field EPR spectra of the fast form of the enzyme isolated by our modified Hartzell–Beinert procedure, the slow form prepared by incubation of the fast form at low pH, the fast form reacted with 100 mM sodium formate, and enzyme isolated by the Volpe–Caughey method are compared in Figure 3. As noted by Baker et al. (1987), the Volpe–Caughey preparations always exhibit the $g' = 12$ resonance, while this signal is absent in the Hartzell–Beinert prepared by using Baker's modification. The spectrum of the slow form is characterized by the $g' = 12$ signal which develops as a result of exposure of the fast enzyme to low pH. Reaction of fast enzyme with sodium formate also produces the $g' = 12$ signal. In contrast to the slow appearance of the signal during the spontaneous production of the slow form, the $g' = 12$ signal develops within minutes following the addition of formate to enzyme buffered at pH 8.1 (vide infra). Addition of TMPD and sodium ascorbate to the formate species (see Materials and Methods) produces the mixed-valence species in which only cytochrome *a* and Cu_A are reduced (Babcock et al., 1976). The $g' = 12$ signal is still present in this form of the enzyme.

A second change in the EPR spectrum of the slow form is the 15–20 upfield shift of the $g = 3$ signal of cytochrome *a*. The $g = 3$ signal of the formate derivative is also slightly shifted upfield, in this case by about 10 G, in partial agreement with the report of Keyhani and Keyhani (1980), who also reported that the $g = 3$ resonance was split by this reagent.

Greenaway et al. (1977) as well as Baker et al. (1987) have shown that the $g' = 12$ signal develops during the purification of the enzyme. Baker et al. (1987) further observed that the formation of this EPR signal and thus the conversion to the slow form are a result of exposure of the enzyme to low pH and low enzyme concentration. We do not observe this $g' = 12$ signal in mitochondrial preparations used as the starting point for enzyme isolated by the Hartzell–Beinert protocol, in confirmation of the original observation of Baker et al. (1987). Slow enzyme evidenced by this EPR signal is therefore not believed to be present, at least not at a significant concentration, in the mitochondrial membrane. However, upon addition of 50–100 mM sodium formate to mitochondrial preparations and incubation on ice for 45 min, the $g' = 12$ signal is formed (Figure 4) in amounts comparable to that seen with purified slow enzyme; mitochondria maintained under the same conditions in formate-free buffer did not develop this EPR signal. Shorter times of incubation have not been examined. The $g' = 12$ signal is not lost by subsequent washing of the mitochondria by cycles of concentration by centrifugation and dilution with formate-free buffer. A loss in intensity at $g = 6$ and a shift to higher field of the $g = 3$ signal seen with purified enzyme are also observed as a result of addition of sodium formate.

The production of the $g' = 12$ signal is exclusive to the formate species; no other reagent that we have examined when added to fast enzyme results in the appearance of the EPR signal. Thus, while the enzyme treated with formamide, formaldehyde, and sodium nitrite exhibits a blue-shifted Soret maximum similar to that found with formate, no $g' = 12$ signal is detected with these alternative reagents.

Sodium nitrite is unique in that it does cause a change in the $g = 3$ region with the single peak characteristic of cytochrome *a* being converted into two peaks (Figure 5); this feature is apparent as a marked shoulder about 45 G to lower field of the $g = 3$ signal. Higher concentrations of sodium

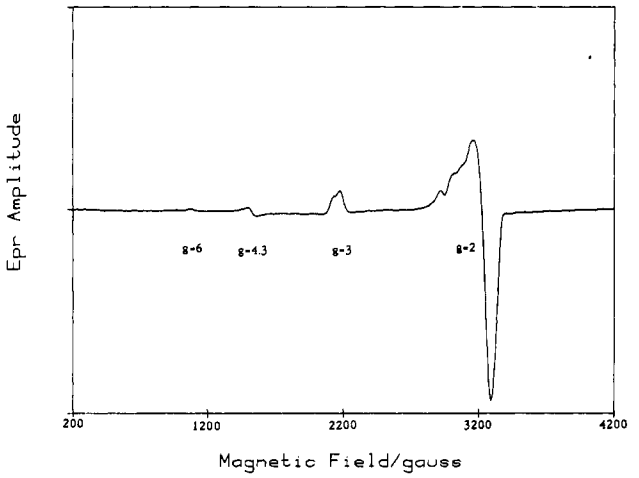

FIGURE 5: EPR spectrum of fast enzyme with sodium nitrite. Fast enzyme (100 μM in heme *a*) reacted with 50 mM $NaNO_2$. The $g = 3$ and $g = 2$ regions demonstrate significant changes, while the low-field region in unchanged.

nitrite (up to 1 M) do not significantly alter the relative intensities of the two EPR peaks near $g = 3$ while the Soret maximum is stable at 420 nm and no $g' = 12$ intensity is noted at these higher nitrite concentrations. Nitrite also causes changes in the $g = 2$ region, producing an extra feature at $g = 2.1$ which is not observed in the spectrum of resting enzyme.

No change in the EPR spectrum of fast cytochrome oxidase was found with enzyme incubated with reagents such as acetate, trifluoroacetate, sulfate, phosphate, and bicarbonate.

Resonance Raman Spectra. RR spectra of the fast and slow forms of cytochrome oxidase as well as the fast enzyme plus formate species reveal that the oxidation- and spin-state markers of cytochromes *a* and a_3 are unchanged (Schoonover et al., 1988). It is therefore evident that cytochrome a_3 is oxidized, high-spin, and six-coordinate in all three of these forms of the enzyme.

One obvious difference in the three spectra in the high-frequency region is the intensity increase at the 1620 cm^{-1} feature and the concomitant loss of intensity near 1650 cm^{-1}, with the 1620 cm^{-1} feature being very intense in the slow enzyme and formate derivative spectra, but very weak in the spectrum of fast enzyme. The origin of these changes has been discussed in detail (Schoonover et al., 1988). The high-frequency spectrum of the formamide derivative is very similar to that of the formate species (J. R. Schoonover, G. Palmer, and W. H. Woodruff, unpublished results).

Both slow enzyme and the formate species demonstrate enhancement of low-frequency modes with 413.1-nm excitation as a result of the shift of the cytochrome a_3 Soret band toward the excitation wavelength. We initially reported that the fast and slow forms exhibited different frequencies for the Fe–His stretch at 220 cm^{-1}. However, recent experiments (J. R. Schoonover, G. Palmer, and W. H. Woodruff, unpublished results) indicate that the shift of the peak assigned to the Fe(III)–N(his) stretch of cytochrome a_3 observed in slow enzyme is in fact due to pH differences in the two samples and not a result of the conversion of the enzyme to the slow form. No shift of this vibration is observed upon adding formamide to fast enzyme at pH 8.1; only enzyme maintained at lower pH exhibits the shift of the Fe–His mode to lower frequency.

Cyanide-Binding Studies. Baker et al. have correlated the amplitude of the slow phase in the reaction with cyanide to the intensity of the $g' = 12$ EPR signal (Baker et al., 1987). Under the experimental conditions used in this study, slow enzyme typically exhibits a $k_{obs} = 0.0001$–0.0002 s^{-1} for the pseudo-first-order reaction with cyanide, while fast enzyme demonstrates values between 0.04 and 0.08 s^{-1}. However, in the presence of sodium formate, fast enzyme shows cyanide kinetics which are comparable to those of slow enzyme with a single-exponential phase; the value of $k_{obs} = 0.0001$ s^{-1}. The addition of formamide or sodium nitrite also induce a single slow phase in the rate of binding cyanide; the values of k_{obs} are 0.001–0.002 and 0.001 s^{-1}, respectively. These latter values are an order of magnitude larger than those of the slow and formate forms of the enzyme, indicating a different effect on the binuclear center.

Formate Binding. Nicholls (1975) found that formate slows the rate of oxygen uptake in a mixture of ascorbate, TMPD, cytochrome *c*, and cytochrome oxidase. He also noted that the inhibition of cytochrome oxidase by formate was much more rapid at the more acidic pH values and interpreted this result as indicating that formic acid is the bound species.

The rate at which sodium formate causes the Soret band to undergo a blue-shift is highly pH-dependent. Thus, at pH 8.1, enzyme treated with 100 mM formate requires 1 h for the blue-shift to be complete while at pH 6.2 these absorbance changes are complete within 2 min. By comparison, the spontaneous conversion of fast enzyme to the slow form produced by incubation at pH 6.2 requires 48–72 h. The rate of the Soret blue-shift induced by formamide is much less pH-dependent, spectral changes being complete in 15–20 min at both pH 6.2 and pH 8.1.

To establish the time scale for the appearance of the $g' = 12$ signal, rapid-freeze EPR studies were performed. In these experiments, 200 μM (in heme *a*) enzyme and 200 mM sodium formate buffered at pH 8.1 were mixed, and the mixture was delivered to an EPR tube; the contents of this tube were then frozen after an appropriate time interval. The EPR data indicate that, at pH 8.1, the $g' = 12$ signal is maximally formed within the first 2 min and production of the $g' = 12$ species is therefore complete before any significant change in the position of the Soret maximum has occurred. No further change in the intensity of the $g' = 12$ signal is observed in spectra obtained at time intervals ranging from 2 min to over 2 h.

The blue-shift of the Soret maximum and the $g' = 12$ EPR signal induced by the addition of sodium formate could not be reversed by procedures expected to remove the formate ion were it loosely bound. Thus, washing the formate derivative by cycles of concentration and dilution with buffer containing no sodium formate has no effect on the position of the Soret absorbance or the presence of the $g' = 12$ signal. Likewise, applying the formate derivative to a Bio-Gel P6 column and eluting with formate-free buffer also do not change these spectral parameters. The Soret maximum remains at 418 nm even when the pH is increased to over 8.0.

The possibility that formate was not removed by these procedures was evaluated as follows. Enzyme was incubated with sodium formate radiolabeled with ^{14}C. This sample was applied to a P6 column and eluted with formate-free buffer; aliquots of the eluate were examined for radioactivity by scintillation counting and for enzyme by absorbance measurements. Figure 6 shows the results of such as experiment. The elution profile demonstrates measurable radioactivity as the enzyme is eluted from the column (inset). Subsequent to the elution of the enzyme, free formate was detected as evidenced by the large increase in radioactivity; free formate and bound formate were well resolved from one another. Clearly, formate is not removed from the isolated enzyme in the oxi-

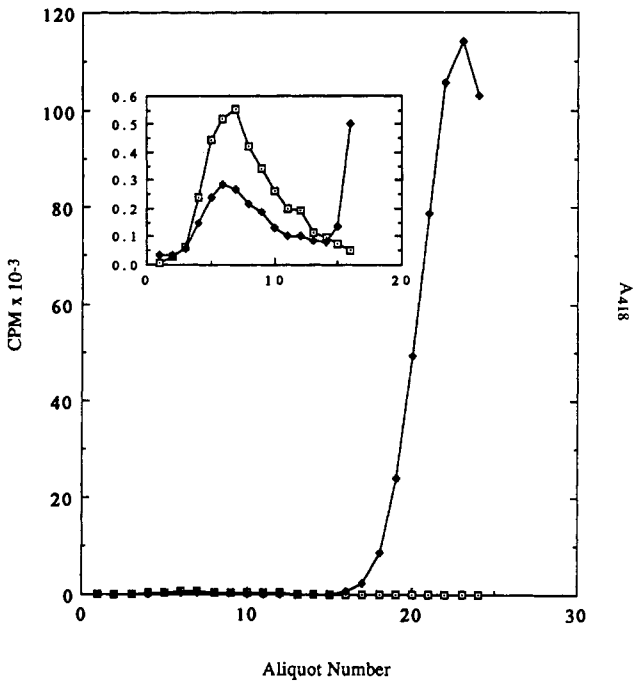

FIGURE 6: Elution patterns for radioactivity and the absorbance at 418 nm for following application of ^{14}C-labeled sodium formate treated cytochrome oxidase to a Biogel P6 column. Black boxes denote radioactivity, and white boxes indicate the absorbance at 418 nm. The inset is an expanded display of aliquots 1–16.

dized state by the methods we have employed. In two separate experiments, the amount of formate bound to the enzyme was found to be 1.3 and 2.3 equiv per aa_3.

Discussion

We have previously demonstrated that the formation of the slow form of cytochrome oxidase is a consequence of a substantial structural change at the binuclear center comprised of cytochrome a_3 and Cu_B (Baker et al., 1987; Palmer et al., 1988; Schoonover et al., 1988). Thus, in the slow form of the enzyme, this site is much less reactive with exogenous ligands as exemplified by cyanide, it exhibits the $g' = 12$ signal in low-temperature EPR spectra, and, as we have recently shown, the values of E, the rhombic zero-field splitting parameter associated with the $S = 2$ binuclear center, of the slow and fast forms are of opposite sign (Day et al., 1989). This change in zero-field splitting is further evidence for a structural modification at the binuclear center. At this point, the relevant objective becomes the identification of the origins of this change and its possible physiological relevance. For instance, can this change be attributed to a specific chemical event or is it the result of a more general structural rearrangement?

In pursuing this objective, we have found that conversion to the slow form can be induced simply by the addition of sodium formate to the fast form; this leads to spectral and kinetic changes which appear to be equivalent to those observed with the slow-reacting form produced spontaneously. Thus, the blue-shift of the net Soret maximum, visible MCD changes, the appearance of the $g' = 12$ EPR resonance, and slow cyanide-binding kinetics obtained after addition of formate to fast enzyme are all characteristic of the slow form of the enzyme prepared by incubation of the fast form at low pH. Certain of these phenomena have been observed previously. Thus, Boelens and Wever (1979) found that addition of formate to mixed-valence enzyme led to the appearance of the $g' = 12$ signal while Keyhani and Keyhani (1980) characterized the properties of resting Yonetani enzyme treated with formate and drew attention to the marked changes in the low-field resonance of cytochrome a^{3+}. In this latter case, the ability of formate to induce the $g' = 12$ signal was obscured by the large intrinsic $g' = 12$ signal of the Yonetani preparation.

While the large blue-shift (6 nm) in the Soret band and the slow cyanide reactivity also imply an alteration in the a_3–Cu_B site for the formate derivative, we have previously obtained Raman evidence that this alteration is not associated with a change in oxidation state, spin state, or coordination number in either slow enzyme or the formate derivative (Schoonover et al., 1988; unpublished data). Furthermore, the RR data provide additional evidence for a significant change in the overall protein conformation, for, upon incubation in D_2O, spectra of fast enzyme exhibit shifts of both the cytochrome a_3 Fe(III)–N(his) and the cytochrome a formyl vibrations which are not observed in the slow enzyme even though spectra were recorded at intervals over a period of several hours; this indicates that these sites on the slow enzyme have decreased accessibility to deuterons. These Raman results were interpreted as indicating that formation of slow enzyme leads to conformational changes which involve both cytochrome a and cytochrome a_3.

There are at least two significant differences between the formate derivative and the slow form obtained spontaneously. First, the spectral changes induced by the addition of formate to rapid enzyme occur quite rapidly, with the formation of the $g' = 12$ EPR signal being complete within 2 min at all pH values while the blue-shift of the net Soret maximum is complete within minutes (pH 6.2) to about an hour (pH 8.1). The decrease in cyanide reactivity is also rapidly established at pH 8 (G. Liao and G. Palmer, unpublished data), thus confirming the previously reported strong correlation between the $g' = 12$ signal and the kinetics of cyanide reactivity (Baker et al., 1987). Second, the position of the Soret maximum of the formate derivative does not vary over as wide a pH range as that of the slow form. Thus, upon increasing the pH from 6.0 to 8.1, the spontaneously produced slow form remains slow as judged by the very low rate of reaction with cyanide and the continued presence of the $g' = 12$ signal, but the wavelength of the Soret peak increases from 416 to 423 nm. By contrast, the formate derivative exhibits less than a 2-nm red-shift of the Soret maximum over the same pH range; a similar weak dependence on pH is also found with fast enzyme (Baker et al., 1987; this work).

Other than formate, we have not found any reagent which, when added to fast enzyme, causes the formation of $g' = 12$ EPR signal and the very slow rate of reaction with cyanide. While several reagents, notably those structurally related to formate, can cause a blue-shift of the a_3 component of the Soret absorption, this shift occurs without any obvious effect on the EPR properties of the enzyme and without the extreme inhibition of the rate of reaction with cyanide. Moreover, those potential heme ligands that have been examined, e.g., fluoride and azide, were found to be ineffective in inducing the transition to the slow form.

This unique property of formate suggests that there is a highly specific binding site for this compound. Our initial attempts to document that using radiolabeled formate suggest that there is one such site per enzyme. However, our data do not exclude the possibility that the formate is bound strongly but nonspecifically though both experiments of Nicholls (1975, 1976), and the highly specific consequences of formate binding we report here make this rather unlikely. Additional experiments to verify this specificity of binding are in progress.

Conversion to the slow form and addition of formate to fast enzyme lead to a small decrease in the low-field g value of cytochrome a. This effect of formate was previously noted by both Boelens and Wever (1979) and Keyhani and Keyhani (1980) with the latter further reporting that the EPR of this feature was split into a slightly resolved doublet upon addition of formate; this apparent splitting was interpreted as a consequence of the conversion of a small amount of cytochrome a_3 to the low-spin form. We do not accept this interpretation for the following reasons: (i) the area under the $g = 3$ EPR feature is unchanged by this treatment; (ii) the amplitude of the Soret MCD is not increased on addition of formate; and (iii) formate causes a blue-shift of the Soret maximum. None of these pieces of data are consistent with the conversion of a fraction of a_3 to the low-spin form. It should be noted that similar EPR shifts can be produced by high concentrations of KCl and, remarkably, simply by changing the gas in equilibrium with the enzyme (Hartzell & Beinert, 1974) and it seems more credible that this change in the $g = 3$ feature reflects some overall change in the structure of the enzyme as we had previously inferred from the effect of D_2O on the Raman spectra of fast and slow enzyme (Schoonover et al., 1988). At the same time, we do find that nitrite produces a splitting of the $g = 3$ signal. Furthermore, Hartzell and Beinert (1974) reported that the line shape of this feature responded inhomogeneously during a redox titration. Whether or not this behavior of cytochrome a is a reflection of an intrinsic inhomogeneity at this stie, or, more interestingly, is a reflection of the heme–heme interaction which is known to be present [see Kojima and Palmer (1983) and references cited therein] remains to be established.

A plausible explanation for the fast to slow transition which we have previously suggested consists of the slow form undergoing a change in the ligand which bridges the a_3 heme and Cu_B (Baker et al., 1987; Palmer et al., 1988). This change could account for the sluggish reactivity of ligands as well as the modification of the magnetic properties of the binuclear center. However, recent infrared results suggest an intriguing alternative.

Yoshikawa and Caughey (1990) have now provided convincing infrared data that cyanide is bound exclusively to copper in the resting, oxidized form of the enzyme. As the EPR spectrum of Cu_A is unaffected by the addition of cyanide, it seems logical that the site of coordination of the cyanide is Cu_B.[3] Previously, Brudwig et al. (1981) had obtained evidence that nitric oxide can also coordinate to Cu_B in resting enzyme for, with some preparations of cytochrome oxidase, addition of this reagent elicits a high-spin heme EPR signal which was interpreted as arising from the heme of cytochrome a_3 following the formation of a diamagnetic Cu_B–NO adduct. There is thus good evidence for the existence of a readily available site for ligation on Cu_B, a site which either is normally vacant or bears a labile ligand. This ligand is denoted denoted L in Figure 7A in which a speculative structure for the binuclear center is depicted. Addition of cyanide (and possibly NO) displaces L, as shown in Figure 7B. In this cyanide derivative, it is proposed (Yoshikawa & Caughey, 1990) that the presence of the cyanide modifies the bond between Cu_B and X, the bridging ligand, and thus, indirectly, the bond between X and the heme iron of cytochrome a_3, leading to the well-known changes in optical and MCD spectra (Babcock et al., 1976; Thomson et al., 1981).

[3] This conclusion is presumably unaffected if Cu_A consists of a binuclear Cu(II)–Cu(I) pair, as has recently been suggested (Kroneck et al., 1990).

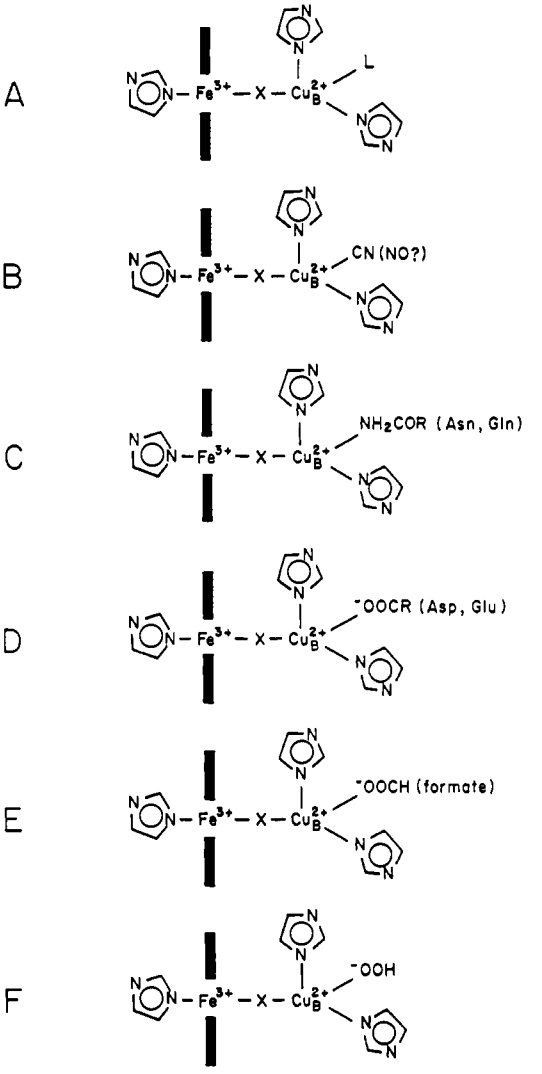

FIGURE 7: Hypothetical structures of the binuclear center of cytochrome oxidase. (A) Structure present in fast enzyme showing cytochrome a_3 edge-on with its proximal histidine group, the presumed but as yet unidentified bridging ligand (X), the postulated weakly bound ligand (L), and two nitrogenous ligands assumed to be imidazolate moieties. (B) shows the displacement of L by cyanide and presumably nitric oxide. (C) speculates that L is an amide function in fast enzyme which is replaced by a different residue in (D) to yield slow enzyme. (E) represents the postulated structure obtained when formate is used to produce the slow form of the enzyme and (F) the hydrogen peroxide adduct.

We now hypothesize that in the rapid conformer of cytochrome oxidase L is an amide function provided, perhaps, by a peptide bond or by either asparagine or glutamine. (Figure 7C). Because amides are weakly coordinating, they should be readily displaced by cyanide, which is a much stronger nucleophile. The fast to slow transition can now be interpreted as a structural change in which L is replaced by a much more strongly bound amino acid function. For example, asparagine might be hydrolyzed to aspartate as shown in Figure 7D, or there may be a conformational change which removes L and brings a different amino acid such as histidine to this coordination site of Cu_B. The higher affinity of the new ligand for Cu_B impedes the reaction of Cu_B with cyanide, and thus the cyanide kinetics typical of the slow form are obtained.

Although the amino acid function which replaces L in the conversion of fast enzyme to slow enzyme (i.e., Figure 7A → Figure 7D) is depicted as carboxylate in Figure 7D, the substantial shift in the Soret maximum of the slow form as the pH is raised from 6 to 8 favors the idea that the coordinating

amino acid residue is histidine rather than the carboxylate-bearing residue shown (Figure 7D). This shift can then be rationalized by invoking the ionization of imidazole to imidazolate as the pH is raised for such an ionization would promote increased charge density at the copper which in turn would release charge to the heme iron; this should result in a red-shift of the optical spectrum.

We propose that addition of formate to the resting enzyme produces a species such as shown in Figure 7E in which L has been replaced by the formate anion. We speculate that this substitution also impedes reaction with cyanide and yields slow kinetics. As formate does not bear an additional ionizable group, the pH sensitivity of the Soret maximum would be eliminated. The existence of a discrete site for formate binding is supported by our finding that approximately 1 equiv of formate remains on the enzyme despite attempts to remove the formate by gel filtration.

Following the logic of Yoshikawa and Caughey (1990), we now argue that the addition of formate to Cu_B will change the nature of the bond between Cu_B and X, the presumptive bridging ligand between copper and iron. This, in turn, can lead to a change in the character of the bonding between X and the iron atom of cytochrome a_3, and it is this change in bonding which we propose to be responsible for the characteristic changes in the optical spectrum, the magnetic susceptibility, and the appearance of the $g' = 12$ EPR signal following addition of formate.

There are three additional pieces of information consistent with the presence of an available site for ligation on Cu_B. First, addition of a variety of reagents, including weak-field and strong-field heme ligands, to resting cytochrome oxidase has negligible to small effects on the optical and EPR properties of the enzyme but dramatically modifies the kinetics of the reaction with cyanide (G. Liao and G. Palmer, unpublished rsults). Second, the hydrogen peroxide adduct of resting enzyme has modified UV-vis and Raman spectra, but the changes in the Raman spectra between 150 and 1200 cm^{-1} are essentially insensitive to replacing the $H_2{}^{16}O_2$ with $H_2{}^{18}O_2$ (G. Palmer, W. A. Oertling, J. S. Schoonover, R. B. Dyer, W. H. Woodruff, unpublished results). This lack of sensitivity to the heavy isotope is not consistent with the direct bonding of H_2O_2 to the heme iron but can be explained if H_2O_2 binds to Cu_B as shown in Figure 7F. Finally, the reaction of H_2O_2 with the fast enzyme under strictly second-order conditions exhibits rigorously first-order kinetics (G. Liao and G. Palmer, unpublished results), a result which is nicely explained if the addition of H_2O_2 requires prior dissociation of a preexisting ligand.

Our observation of the formation of the $g' = 12$ EPR signal in mitochondrial preparations following addition of sodium formate is particularly significant in that we had previously believed that this signal was uniquely an artifact of the isolation procedure (Baker et al., 1987). The $g' = 12$ signal has not been previously observed in EPR spectra of mitochondria, and Baker et al. (1987) found that low enzyme concentration and exposure of the isolated enzyme to low pH were the major causes of the structural modification leading to the appearance of this $g' = 12$ EPR resonance. Since this change in the magnetic properties of the enzyme—and by inference the conversion to slow enzyme—can be induced in mitochondria by the addition of formate, we conclude that the postulated Cu_B ligation site is still present when the enzyme is in its natural environment. It thus seems reasonable to anticipate that this site has an important physiological role.

It is distinctly relevant that Woodruff et al. (1991) have recently obtained kinetic data for the binding of carbon monoxide to fully reduced soluble cytochrome oxidase over a wide temporal range which was interpreted as showing that a site on Cu_B is used during the passage of CO from solvent to the heme iron of cytochrome a_3, and we have speculated (Woodruff et al., 1991) that this route is also followed during reaction with oxygen. We also concluded that upon binding of CO to Cu_B a ligand is transferred to cytochrome a_3. It seems plausible that this site is identical with the one that we call L and raises the possibility that this coordination site loses and regains an endogenous ligand during reduction and subsequent reoxidation. Such behavior can be inferred from the data of Nicholls (1975, 1976), who reported that full reduction followed by oxygenation of submitochondrial particles previously incubated with formate reverses formate inhibition, implying that formate is released upon reduction. Additional support for this idea is provided by Baker et al. (1987), who found that it is possible to convert the slow conformer back to the fast conformer for a limited time by a cycle of reduction and reoxidation. Rapid cyanide reactivity was restored by this treatment while the $g' = 12$ EPR signal was eliminated. However, this form of the enzyme spontaneously reverts back to the slow form after several hours as judged by the loss of the rapid reaction with cyanide and the appearance of the $g' = 12$ EPR signal.

The likelihood that there is ligand dissociation and reassociation at the site of oxygen reduction as the enzyme is cycled through its redox states has clear mechanistic implications and may have a fundamental bearing on the proton-pumping capabilities of this enzyme, especially in view of the recent proposal (Wikstrom, 1989) that the binuclear center is critically involved in proton pumping. While the structural change implied by our studies is unlikely to be the primary proton-translocating event, it may well have relevance to the requirement for a "proton gate" which is inherent in general models for proton translocation, and consequently the increasing evidence for the involvement of Cu_B in the ligand reactivity of the binuclear center and the consequential structural changes that we believe to occur clearly warrants further examination and is being pursued.

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Relative Affinities of Divalent Polyamines and of Their N-Methylated Analogues for Helical DNA Determined by ^{23}Na NMR†

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ABSTRACT: Interactions of divalent polyamines with double-helical DNA in aqueous solution are investigated by monitoring the decrease in ^{23}Na NMR relaxation rates as NaDNA is titrated with $H_3N^+-(CH_2)_m-^+NH_3$, where m = 3, 4, 5, or 6. Analogous measurements are made for the same homologous series of methylated polyamines (methonium ions). The dependence of the ^{23}Na relaxation rates on the amount of added divalent cation (M^{2+}) is analyzed quantitatively in terms of a two-state model. The sodium ions are assumed to be in rapid exchange between a "bound" state, where they are close enough to DNA so that it affects their relaxation rate, and a "free" state in bulk solution, where their relaxation rate is the same as in solutions containing no DNA. The distribution of Na^+ and M^{2+} between these states is described quantitatively in terms of an ion-exchange parameter: $D_M \equiv (p_B^M)(1-p_B^{Na})^n/(p_B^{Na})^n(1-p_B^M)$, where p_B^{Na} and p_B^M are the fractions of Na^+ and M^{2+} that are close enough to DNA to be considered bound (by the NMR criterion), and n is the number of sodium ions displaced from DNA by the binding of one M^{2+} ion. For each of the polyamines and methonium ions investigated here, equations derived from this two-state model yield acceptable fittings of the titration curves if r_{Na}^0, the number of sodium ions bound per DNA phosphate when no competing cations are present, is assigned a value between 0.6 and 1.00. Within this range, changing the value assigned to r_{Na}^0 does change the best-fitted values of D_M determined for these polyamines (D_H) and for the methonium ions (D_{Me}) but does not alter the following conclusions about the trends in these parameters. (1) For polyamines and methonium ions of the same m, D_H exceeds D_{Me} by factors that are significantly larger for m = 3 and 4 than for m = 5 and 6. (2) D_H for m = 3 and 4 is larger than D_H for m = 5 and 6. (3) D_{Me} for m = 3 and 4 is smaller than D_{Me} for m = 5 and 6.

Polyamines interact with polyanionic nucleic acids and with membranes in vivo [cf. Tabor and Tabor (1985)]. To clarify in quantitative terms the biological significance of these interactions, their molecular and thermodynamic consequences have been extensively investigated in vitro. Thermodynamic and spectroscopic measurements have provided some information about competitive interactions of polyamines and inorganic cations with DNA in aqueous solution (Braunlin et al., 1982, 1986; Burton et al., 1981; Padmanabhan et al., 1988; Thomas & Bloomfield, 1984; Thomas et al., 1985; Thomas & Messner, 1988; Vertino et al., 1987). These studies indicate that the concentrations and types of polyamines and small inorganic cations (like Na^+) in a solution containing DNA have profound effects on its conformational stability and on the extent to which it forms complexes with proteins or other

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