Chemical Modification of the Cu_A Center in Cytochrome *c* Oxidase by Sodium *p*-(Hydroxymercuri)benzoate†

Jeff Gelles and Sunney I. Chan*

Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125

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ABSTRACT: Cytochrome *c* oxidase contains a copper ion electron-transfer site, Cu_A, which has previously been found to be unreactive with externally added reagents under conditions in which the protein remains structurally intact. We have studied the reaction of cytochrome oxidase with sodium *p*-(hydroxymercuri)benzoate (pHMB) and found that the reaction proceeds, under appropriate conditions, to give an excellent yield of a particular derivative of the Cu_A center that has electron paramagnetic resonance and near-infrared absorption spectroscopic properties which are distinctly different from those of the unmodified center. Spectroscopic and chemical characterization of the other metal ion sites of the enzyme reveals little or no effect of the pHMB modification on the structures of and reactions at those sites. Of particular interest is the observation that the modified enzyme still displays a substantial fraction of the native steady-state activity of electron transfer from ferrocytochrome *c* to O_2. Although the modified copper center retains the ability to receive electrons from the powerful reductant $Na_2S_2O_4$ and to transfer electrons to O_2, it is not significantly reduced when the enzyme is treated with milder (higher potential) reductants such as NADH/phenazine methosulfate or the physiological substrate ferrocytochrome *c*. Cu_A exhibits many spectroscopic and chemical properties which make it highly atypical of cuproprotein active sites; the singular nature of this site has prompted speculation about the importance of the structural peculiarities of this metal ion center in the catalytic cycle of the enzyme. In this work, we demonstrate that the unusual features of this site are *not* prerequisites for competent catalysis of electron transfer and O_2 reduction by the enzyme. Specifically, these observations support the model in which a secondary electron-transfer pathway not involving Cu_A exists between the cytochrome *c* and oxygen binding sites which can function at a rate at least 20% of that in the native enzyme.

Cytochrome *c* oxidase catalyzes the final step of the mitochondrial electron transport chain in which electrons derived from the oxidation of ferrocytochrome *c* are consumed in the reduction of oxygen to water. Each molecule of the enzyme contains two copper ions and two hemes. Three of these four metal centers are known to play critical roles in the catalytic cycle: one of the hemes (referred to as Fe_a)[1] is the primary electron acceptor from cytochrome *c*, and its location within the enzyme is presumed to be near that of the cytochrome *c* binding site (Malmström, 1980) [although for an alternative view see Capaldi et al. (1983)]; the other heme (Fe_{a_3}) and one of the copper ions (Cu_B) together make up the binuclear site that binds O_2 and O_2-derived intermediates while they undergo a complex sequence of chemical conversions that ultimately result in the synthesis of two H_2O molecules. The O_2-binding site is thought to reside in a hydrophobic environment inside the protein (Fiamingo et al., 1982; Alben et al., 1981), and is located a substantial distance (approximately 20 Å) away from Fe_a (Brudvig et al., 1984). The enzyme must therefore contain an electron-transfer pathway that connects the two sites and facilitates the rapid movement of electrons during turnover.

The role played by the fourth metal center, the low-potential copper ion site (Cu_A), is less well understood. Pioneering studies of the reduction and oxidation kinetics of this site during the reaction of the enzyme with ferrocytochrome *c* or with O_2 by Gibson & Greenwood (1965) (using optical spectroscopy) and by Beinert & Palmer (1964a) [using electron paramagnetic resonance (EPR) spectroscopy] revealed rapid reaction rates similar to those observed for Fe_a. The results of these and more recent kinetic studies have in general been interpreted in terms of a catalytic mechanism in which Cu_A is located between Fe_a and the O_2-binding site in the electron-transfer pathway within the enzyme and serves as a redox mediator facilitating electron transfer from the former to the latter (Gibson & Greenwood, 1965). It should be noted, however, that more elaborate (Clore et al., 1980) or completely different (Wikström et al., 1981) mechanisms have also been proposed. Since it is difficult to distinguish between these alternative mechanisms on the basis of kinetic data alone, other types of studies are required to provide additional insight.

An approach that has proven to be particularly valuable in elucidating the functional role of individual metal centers in multisite metalloproteins is the preparation of enyzme derivatives in which the structure of an individual metal center is perturbed by chemical modification. Chemical, spectroscopic, and kinetic analyses of these derivatives can yield information about the involvement of that center in particular parts of the

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[1] Abbreviations: pHMB, sodium *p*-(hydroxymercuri)benzoate; pCMB, sodium *p*-(chloromercuri)benzoate; EPR, electron paramagnetic resonance; PMS, phenazine methosulfate; Fe_a, the low-potential heme site of cytochrome *c* oxidase; Cu_A, the low-potential copper site of cytochrome *c* oxidase; Fe_{a_3}, the high-potential heme site of cytochrome *c* oxidase; Cu_B, the high-potential copper site of cytochrome *c* oxidase; Tris, tris(hydroxymethyl)aminomethane.

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catalytic cycle. Studies on *Polyporus* laccase, for example, have shown that fluoride ions bind specifically to the type 2 copper of this enzyme. This structural change decreases the reduction potential of the copper center and inhibits enzyme turnover (Malkin et al., 1968; Reinhammar, 1972).

The oxygen binding site of cytochrome oxidase also reacts readily with certain externally added reagents, and these reactions have been used extensively as structural probes of the chemical structure and reactivity of this binuclear site (Lemberg, 1969; Blair et al., 1983). Cu_A, in contrast, appears to be inaccessible to added reagents; although chemical modification of this site has been reported, this has only been accomplished under conditions that cause denaturation and/or complete inhibition of the enzyme (Beinert & Palmer, 1964b; Weintraub & Wharton, 1981; Chan et al., 1979). Even though no reagents are known that produce chemical changes at the Cu_A site in the intact enzyme (except for the reversible, one-electron redox transformations associated with electron transfer through the enzyme), a great deal of information about Cu_A has been obtained through extensive study of the spectroscopic properties of this center. A number of these features are unique among the copper proteins and undoubtedly reflect an unusual ligation structure at the site. Some of the amino acid side chains which serve as ligands to Cu_A were recently identified by Chan and co-workers (Stevens et al., 1982), who used a variety of magnetic resonance techniques to study yeast cytochrome *c* oxidase that had been metabolically labeled with isotopically substituted amino acids. These studies demonstrated the presence of at least one histidine and at least one cysteine ligands at the site. Data from subsequent experiments suggest that a second cysteine is also associated with the Cu_A site (Martin, 1985; C. T. Martin, C. P. Scholes, and S. I. Chan, unpublished results).

It has long been suspected that one or more thiol groups play important roles in the catalytic turnover of cytochrome oxidase. The effect on the enzyme's structure and activity of chemical modification with thiol-reactive compounds, particularly mercury compounds, has been extensively investigated (Beinert & Palmer, 1964b; Tsudzuki et al., 1967; Williams et al., 1968; Wainio & Nikodem, 1973; Chan et al., 1979; Brudvig & Chan, 1979; Mann & Auer, 1980; Darley-Usmar et al., 1981; Bickar et al., 1984). It has been suggested that the nonpolar mercurial reagents $HgCl_2$, CH_3HgCl, and CH_3CH_2HgCl, which at low concentrations cause partial inhibition of oxidase turnover, do so by chemically modifying cysteine ligand(s) to a (unspecified) copper center in the enzyme (Darley-Usmar et al., 1981); however, no evidence of such a modification was detected in EPR studies of CH_3CH_2HgCl-treated cytochrome oxidase (Mann & Auer, 1980). In this work, we report conditions under which the thiol chemical modification reagent sodium *p*-(hdyroxymercuri)benzoate (pHMB) can be used to prepare a derivative of cytochrome *c* oxidase in which Cu_A has been converted from its native structure into a new, spectroscopically distinctive form. Characterization of the spectroscopic properties of the enzyme derivative and of its reactions with cytochrome oxidase substrates and inhibitors reveals little or no change in the structural and chemical properties of Fe_a and the oxygen binding site; the observed changes which accompany the modification reaction are confined to Cu_A. We further show that the modification of Cu_A is accompanied by only a *partial* loss of the overall cytochrome *c*-to-O_2 electron-transfer activity. The residual activity is unlikely to involve electron transfer through Cu_A since there is no detectable reduction of Cu_A^{2+} by substrate under the conditions employed. As these results suggest that Cu_A is not required for simple electron-transfer turnover of the enzyme, we discuss the possible roles that this center may play in catalysis by the enzyme.

Materials and Methods

pHMB, *p*-(chloromercuri)benzoic acid (pCMB), phenazine methosulfate (PMS), NADH, Tween 20, and Tween 80 were purchased from Sigma; mercuric chloride was from Mallinckrodt, and methylmercuric chloride and ethylmercuric chloride were from Alfa. All anaerobic work was performed under 1 atm of argon gas which had been scrubbed of residual oxygen by bubbling the gas through a solution of 0.1 M vanadium(II) in 2 N HCl. Nitric oxide (Matheson) was passed through a cold trap before use (Brudvig et al., 1980); research-grade carbon monoxide (99.99%; Matheson) was used without further purification.

Cytochrome *c* oxidase was purified from beef heart mitochondria by the method of Hartzell & Beinert (1974) and was stored frozen at −80 °C until used. The enzyme preparation used in these studies had a specific activity of 9.0 nmol of O_2 min^{-1} (μg of protein)$^{-1}$ and contained 8.0 nmol of heme *a* (mg of protein)$^{-1}$.

Protein Concentration Measurements. Protein concentrations were assessed by a modification of the Lowry procedure which includes 1% sodium dodecyl sulfate to solubilize integral membrane proteins (Markwell et al., 1978). This technique eliminates interference from nonionic detergents (Cadman et al., 1979). A bovine serum albumin standard was used routinely to check the linearity of the assay; however, except for the measurements of the specific activity and the heme-to-protein ratio reported above, only determinations of the *relative* concentrations of cytochrome oxidase protein are used in this work.

Preparation of pHMB-Modified Cytochrome Oxidase. A 10-mL sample of 22 μM cytochrome *c* oxidase in 50 mM Tris-Cl^-, pH 7.7, 50 mM NaCl, and 0.5% Tween 20 was saturated with pHMB by incubation with 18 mg of this compound with periodic stirring at 20 °C. The reaction time was 25 h unless otherwise specified. The mixture was then cooled to 4 °C and centrifuged at 15000 rpm in a Sorvall SS-34 rotor for 10 min to remove undissolved pHMB. In order to remove unreacted pHMB from solution, the supernatant was transferred to a stirred pressure ultrafiltration apparatus (Amicon 8010 cell with an XM300 membrane) at 4 °C and subjected to three cycles of concentration to 1 mL under 10 psig of N_2 gas followed by redilution to 10 mL with fresh buffer. Separate control experiments demonstrated that essentially all of the cytochrome oxidase but only a small fraction of the Tween 20 is retained by the ultrafiltration membrane under these conditions. After a final concentration step, the enzyme solution was frozen in liquid nitrogen and stored at −80 °C for future use. Samples identified "unmodified control" were prepared exactly as described above except that pHMB was not included in the reaction mixture.

No precipitation or turbidity was observed in samples prepared by the above procedure; however, excessive light scattering was observed in samples from preliminary experiments in which the reaction with pHMB was conducted at a concentration of enzyme approximately 10-fold larger than that described above.

Reaction with Reductants and with Oxygen. For the single-turnover reaction studies, enzyme samples were placed in stopcock-equipped spectrometer cells and were made anaerobic by repeated cycles of evacuation, filling with purified argon, and agitation. Additions of NADH/PMS or sodium ascorbate were accomplished by anaerobic syringe techniques. Additions

of sodium dithionite were conducted by the method of Beinert and co-workers (Tsibris et al., 1968; Orme-Johnson & Beinert, 1969), except that the reductant capillary was placed unsealed in the sample cell sidearm and the entire apparatus was evacuated as a unit. Samples reduced by any of these techniques were reacted with oxygen by replacing the atmosphere over the enzyme solution with 1 atm of air and agitating the cell.

For the EPR spectroscopic studies at −20 °C of enzyme samples reduced by cytochrome *c*/ascorbate, the reaction mixture contained 110 μM cytochrome oxidase, 120 μM cytochrome *c*, and 520 μM sodium ascorbate; the reaction was conducted at 0 °C for 70 min. Conditions for all other reactions with reductants are specified in the figure legends.

Enzyme Activity Measurements. Cytochrome *c* oxidase activity was assayed by observing the rate of O_2 consumption with a YSI Model 53 polarographic oxygen sensor. The measurements were made at 30.0 °C in 3 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80, 15 mM sodium ascorbate, 51 μM cytochrome *c*, and less than 0.1 μM cytochrome *c* oxidase. Neither the native nor the pHMB-modified enzyme preparations displayed any detectable ascorbate oxidase activity when assayed in the absence of added cytochrome *c*.

EPR Spectroscopy. Samples for EPR spectroscopy were equilibrated with 1 atm of argon immediately before freezing to eliminate signals from O_2. EPR spectra were recorded on a Varian E-line Century Series X-band spectrometer, the output signal of which was digitized by a 12-bit analog-to-digital converter interfaced to a minicomputer. Samples were maintained at −20 °C with a nitrogen gas flow system (Varian V4540), at 77 K by immersion in liquid nitrogen, or at 10 K with a helium gas flow system (Air Products Heli-Tran). All combinations of sample temperature and microwave power employed in this study were checked to ensure that they did not cause saturation of the Cu^{2+} EPR signal being observed. Spectra were recorded at the lowest instrument time constant consistent with efficient data sampling; additional postacquisition filtering was applied to some spectra (Ernst, 1966; Savitzky & Golay, 1964). All spectra were corrected for instrument response by subtracting the spectrum of an equal volume of water.

EPR Integration and Simulation. EPR powder spectra were simulated by summing a large number (greater than 8000) of Gaussian envelopes computed for various powder angles. Line positions were calculated assuming coincident **A** and **g** tensors (Atherton, 1973), and line intensities were scaled by $1/g$ (Aasa & Vänngård, 1975).

EPR signal intensities were measured by numerical integration of all or a portion of the signal as described by Aasa & Vänngård (1975). In cases where the second integral of the entire signal was calculated, the position of the integration base line was determined by requiring the first integral to be zero. For the integration of the high-spin ferric heme signals, the above method could not be used because of the presence of interfering Cu^{2+} and low-spin heme signals in the $g = 2$ region. In this case, the line shape of the $g = 6$ region of the signal was simulated (with g_z assumed to be 2.0), and the simulated spectrum was used as a standard for the partial double integration of the experimental spectrum as described by van Gelder & Beinert (1969). The integrals were corrected for the distribution of population among the sublevels of the $S = {}^5/_2$ spin system assuming a zero-field splitting parameter (D) of 10 cm^{-1} (Stevens et al., 1979; Aasa & Vänngård, 1975).

Optical Spectroscopy. Optical absorption spectra were measured at 4 or 20 °C in a Beckman Acta CIII spectrophotometer and were recorded on a Spex Industries SCAMP SC-31 data processor. Spectra were corrected for instrument response by substracting the spectrum of an equal volume of water.

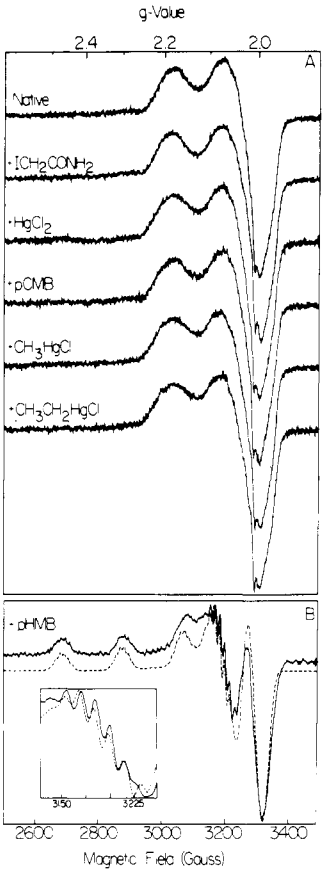

FIGURE 1: EPR signal from the Cu_A^{2+} site after treatment of cytochrome oxidase with thiol modification reagents. (A) Reactions with reagents previously reported to modify cytochrome oxidase cysteine residues. Treatment with the indicated reagent was conducted under the conditions described in the specified reference, with the exception of the detergent used, which was 0.5% Tween 20 in all cases. (Trace 1) Untreated cytochrome *c* oxidase. (Trace 2) Treated with iodoacetamide (Darley-Usmar et al., 1981). (Trace 3) Treated with $HgCl_2$ (Darley-Usmar et al., 1981). (Trace 4) Treated with pCMB (Tsudzuki et al., 1967). (Trace 5) Treated with methylmercuric chloride (Bickar et al., 1984). (Trace 6) treated with ethylmercuric chloride (Mann & Auer, 1980). (B) (Solid trace) Reaction with pHMB under conditions described in this work (see Materials and Methods). (Dashed trace) Simulated EPR powder spectrum calculated for an $S = {}^1/_2$ spin system ($g_x = 2.046$, $g_y = 2.084$, $g_z = 2.226$) with hyperfine coupling to a natural abundance mixture of ^{63}Cu and ^{65}Cu ($I = {}^3/_2$) nuclei and superhyperfine coupling to three equivalent ^{14}N ($I = 1$) nuclei (for Cu, $A_x = A_y = 0$, A_z (^{63}Cu) = 555 MHz, A_z (^{65}Cu) = 595 MHz; for N, $A_x = 24.0$ MHz, $A_y = 43.7$ MHz, $A_z = 40.0$ MHz). (Inset) Magnified view of the spectral region exhibiting the ^{14}N superhyperfine splittings. Spectrum acquisition conditions: sample temperature 77 K; power 4 mW; frequency 9.28 GHz; modulation amplitude 10 G.

RESULTS

Effects of Thiol Modification Reagents on the Spectroscopic Properties of Cu_A. The EPR spectra of native cytochrome *c* oxidase and a number of its previously characterized sulfhydryl reagent derivatives are shown in Figure 1A. Under the spectrometer conditions used, the observed EPR spectrum arises almost exclusively from the oxidized Cu_A center, and it is rather atypical of Cu^{2+} EPR spectra particularly in that it does not display resolved Cu hyperfine splittings (Aasa et

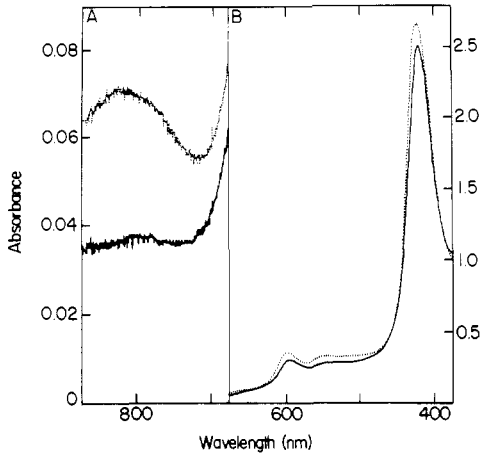

FIGURE 2: Comparison of the near-infrared and visible spectra of native and pHMB-modified cytochrome oxidase. Absorption spectra of a sample of cytochrome oxidase taken before (dotted trace) and after (solid trace) reaction with pHMB under the conditions described under Materials and Methods.

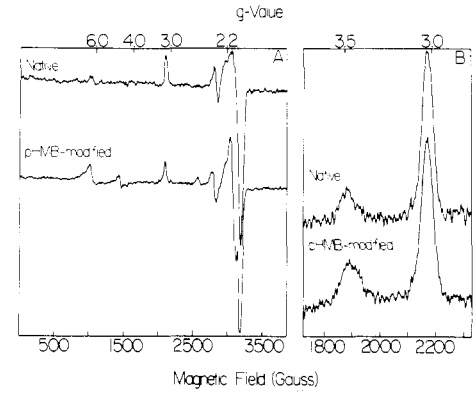

FIGURE 3: Comparison of the low-temperature EPR spectra of native and pHMB-modified cytochrome oxidase. (Top traces) Cytochrome oxidase (unmodified control). (Bottom traces) pHMB-modified cytochrome oxidase. Conditions: sample temperature 10 K; power 0.2 mW; frequency 9.18 GHz. (A) Samples equilibrated with 1 atm of argon. Modulation amplitude: 10 G. (B) Samples containing 3 mM KCN equilibrated with 1 atm of nitric oxide. Modulation amplitude 20 G.

al., 1976; Hoffman et al., 1980; Stevens et al., 1982). The shape of the EPR signal is unchanged by treatment of the enzyme with CH_3CH_2HgCl [as was reported by Mann & Auer (1980)], with CH_3HgCl, or with $HgCl_2$. Cu^{2+} EPR spectra are quite sensitive to changes in the ligands or ligation geometry of the metal center (Peisach & Blumberg, 1974); this observation therefore argues strongly that the reactions of cytochrome oxidase with these reagents do not involve chemical modification of any ligands to Cu_A. The spectrum is also unchanged by treatment of the enzyme with pCMB under conditions shown by Tsudzuki et al. (1967) to label all but one or two of the approximately 14 thiol groups present in one molecule of the beef heart enzyme. If, in contrast, the enzyme is reacted with a high concentration of pHMB[2] for a much longer period of time (see Materials and Methods), a derivative that has a distinctly different EPR spectrum is formed (Figure 1B). This spectrum, which resembles those of the type 2 copper proteins (Vänngård, 1972), is well fitted by a computer-simulated Cu^{2+} EPR powder spectrum which includes a strong ($A_z \simeq 590$ MHz) hyperfine coupling to the copper nucleus as well as superhyperfine interactions with three ^{14}N nuclei. Like the EPR signal from Cu_A in the native enyzme, this signal is not modified by treatment with a low (33 μM) concentration of EDTA, and the species from which it originates cannot be removed from the enzyme by dialysis. We have compared the integrated intensity of the Cu^{2+} EPR absorption spectrum (measured under the same conditions as those in Figure 1) of the pHMB-modified enzyme with that of an unmodified control sample. If the signal from the unmodified enzyme is taken to represent 0.8 mol of Cu_A^{2+}/mol of enzyme (Hartzell & Beinert, 1974), then the intensity of the signal after treatment with pHMB corresponds to 1.1 mol of Cu^{2+}/mol of enzyme. The observed spectroscopic change is therefore more consistent with the conversion of the Cu_A site to a structurally different form than it is with the development of an additional signal from the normally EPR-undetectable Cu_B site.

Comparison of the optical absorption spectra of the native and pHMB-modified enzyme samples provides further evidence of a chemical change at the Cu_A center. Native cytochrome oxidase shows a broad absorption band in the near-infrared region, centered at 830 nm. The band arises from the Cu_A^{2+} site and has been assigned to a charge-transfer transition between the cupric ion and a cysteine ligand (Beinert et al., 1980; Blair et al., 1983). This absorption is completely abolished when the enzyme is reacted with pHMB (Figure 2A). In contrast, only small intensity changes are observed in the visible region, the spectral features of which are largely due to the two heme chromophores of the enzyme (Figure 2B).

Fe_a and the Oxygen-Binding Site in the pHMB-Modified Enzyme. The foregoing observations show that, under appropriate conditions, the treatment of cytochrome oxidase with pHMB causes a structural alteration of the Cu_A site; however, they do not clearly indicate whether the other two electron-transfer sites are also affected by this treatment or whether the changes in the heme spectra are merely indicative of a minor perturbation in the environments of these chromophores. Moreover, it is important to ascertain whether the modified protein is still largely in its native conformation or whether it has been partially or completely unfolded.

In order to more thoroughly characterize the states of the iron centers Fe_a and Fe_{a_3} in the pHMB-modified enzyme, we examined the EPR signals from these centers at low temperature (10 K). The g values of ferric heme complexes are very sensitive to changes in the identity of the axial ligands to the iron (Peisach, 1978). The native enzyme contains a signal due to Fe_a^{3+} at $g_z = 3.0$; an identical peak is observed in the spectrum of the modified enzyme (Figure 3A). Both spectra also contain an Fe_a^{2+} peak at $g_y = 2.2$, although in the case of the pHMB-modified enzyme this is partially obscured by the $M_I = {}^1/_2$ parallel hyperfine component of the signal from the modified Cu_A center. Integration of the $g = 3.0$ signals from two similar enzyme preparations revealed that the modified enzyme retains 87% (after adjusting for the relative protein concentrations of the two samples) of the Fe_a signal intensity in the native enzyme.

Figure 3A shows that the modification reaction is accompanied by the appearance of EPR signals in the $g = 4$ and $g = 6$ regions. Signals at $g = 4$ are frequently seen in cyto-

[2] The protein chemical modification reactions of pHMB and pCMB are essentially identical because both compounds rapidly form the same equilibrium mixture of chemical species when dissolved in neutral aqueous solutions containing millimolar concentration of Cl^- (Boyer, 1954; Webb, 1966). It is therefore likely that the differing effects of the pCMB treatment described by Tsudzuki et al. (1967) and of the pHMB treatment described in the present work arise from the differences between the reaction times and reagent concentrations used in the two sets of experiments rather than from chemical differences between the pCMB and pHMB reagents.

Table I: Catalytic Activity of pHMB-Modified and Unmodified Samples of Cytochrome *c* Oxidase

sample no.	sample type	incubation time (h)[a]	relative protein concn	activity (nmol of O_2 min^{-1}) (mean ± SD)[b]	relative specific activity
1	pHMB modified	24	29.7	22.7 ± 1.6	0.76 ± 0.05
2	pHMB modified	36	24.5	11.9 ± 2.4	0.48 ± 0.10
3	unmodified control	0	16.7	55.2 ± 0.4	3.30 ± 0.02
4	unmodified control	36	25.9	82.2 ± 1.7	3.17 ± 0.06

[a] Incubation time at 20 °C; see Materials and Methods. [b] Mean of three determinations.

chrome oxidase preparations and are thought to arise from low concentrations of contaminants containing non-heme Fe^{3+} ions (van Gelder & Beinert, 1969). The larger signal in the $g = 6$ region is of some concern since axial or rhombic EPR signals with $g_x \simeq g_y \simeq 6$ and $g_z \simeq 2$ are characteristic of high-spin ferric hemes (Wertz & Bolton, 1972). For the bottom spectrum in Figure 3A, however, the integrated intensity in the $g = 6$ signal corresponds to only 3% of the number of Fe^{2+} centers that give rise to the $g = 3$ signal in the same spectrum. This signal may originate in a minor contaminant in the enzyme preparation or may arise from denaturation of a small fraction of the cytochrome oxidase present, but it clearly does not represent a structural alteration at a significant fraction of the oxidase heme sites.

Under ordinary conditions, the Fe_{a_3} and Cu_B centers in native cytochrome *c* oxidase do not exhibit EPR signals (as would ordinarily be expected from Fe^{2+} and Cu^{2+} centers) because they are closely apposed at the oxygen binding site in such a way that a strong exchange interaction exists between the unpaired electrons of the two metal ions (van Gelder & Beinert, 1969; Griffith, 1971). The fact that the reaction of the enzyme with pHMB induces only an insubstantial signal at $g = 6$ and no other new EPR signals that could be assigned to ferric hemes is therefore consistent with the presence of an intact O_2-binding site in the modified enzyme.

More direct information about the structure of the oxygen-binding site is provided by the EPR spectra of native cytochrome oxidase and the pHMB derivative after incubation with nitric oxide and KCN (Figure 3B). $Fe_{a_3}^{3+}$ readily binds CN^- ions, and when NO is also present, the latter binds to Cu_B and breaks the ferromagnetic coupling (Kent et al., 1983) between this center and Fe_{a_3}–CN, thereby rendering the Fe_{a3}^{3+}–CN center EPR visible (Stevens et al., 1979; Brudvig et al., 1981). The peak position observed for this $g = 3.5$ cyanocytochrome a_3 signal and its intensity relative to the amount of protein in the sample are identical in the native and the pHMB-modified samples (*g* values: native, 3.49; modified, 3.47; intensity ratio modified:native = 1.1:1). The above data, taken together, constitute evidence that both Fe_{a_3} and Fe_a are not significantly affected by pHMB treatment under the same conditions in which this reagent completely converts Cu_A into a new, spectroscopically distincitve form.

Catalytic Turnover of the pHMB-Modified Enzyme. We have assessed the catalytic competence of the enzyme containing the modified Cu_A center by measuring its specific activity of cytochrome *c* driven O_2 reduction under pseudo-steady-state conditions. Table I shows that the reaction with pHMB is accompanied by an approximately 5-fold decrease in the activity. As judged by spectroscopic criteria, the pHMB-modified oxidase preparation is virtually free of enzyme molecules containing an unmodified Cu_A site. By comparing the peak height of the near-infrared absorptions ($A_{830} - A_{900}$ from the data in Figure 2) in the control and pHMB-modified samples, we conclude that the modified enzyme contains less than 4% of the original amount of intact Cu_A chromophore. A similar analysis of the EPR spectra of Figure 1 reveals no detectable intensity above the base line from the

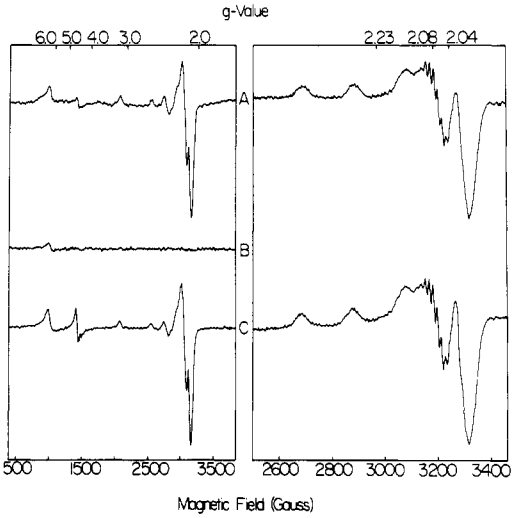

FIGURE 4: Redox behavior of Fe_a and the EPR-visible copper center in pHMB-modified cytochrome oxidase: reduction by sodium dithionite and reoxidation by O_2. (A) Oxidized pHMB-modified cytochrome oxidase (approximately 140 μM). (B) Same sample after anaerobic reduction for 10 min with 1.1 mM $Na_2S_2O_4$. (C) Same sample after subsequent reoxidation with air. Left side spectra conditions: same as for Figure 3A; temperature 9–10 K. Right side spectra conditions: temperature 77 K; power 4.0 mW; frequency 9.26 GHz; modulation amplitude 10 G.

Cu_A^{2+} $g_z = 2.18$ signal in the region of 2975 G. The bulk of the specific activity remaining after pHMB modification (approximately 20% that of the native enzyme) must therefore be attributed to enzyme molecules containing the modified Cu_A center; the specific activity of the modified oxidase preparation is too large to be accounted for by the small amount of native oxidase which might still be present. The observation of residual activity thus implies that the modified enzyme sample contains a substantial number of molecules which possess functionally intact cytochrome *c* binding and O_2-reduction sites and that these sites are connected by an electron-transfer pathway which is at least one-fifth as effective as that in the unmodified control enzyme.

Reduction of the pHMB-Modified Enzyme by Sodium Dithionite and Reoxidation by O_2. It is unclear from the measurements of turnover kinetics whether the modified Cu_A center participates in the electron-transfer process or whether the reactions occur independently of it. However, the effect of reductants and oxidants on the EPR spectrum of the modified enzyme provides some clues about the role that the modified Cu_A center plays in the redox behavior of this species. When an anaerobic sample of native cytochrome *c* oxidase is treated with the powerful reductant sodium dithionite ($Na_2S_2O_4$), all four of its metal centers are reduced, and the EPR signals from Fe_a^{3+} and Cu_A^{2+} are consequently eliminated. The corresponding EPR signals from the pHMB-modified enzyme (Figure 4A) are also removed by $Na_2S_2O_4$ treatment (Figure 4B). As would be expected, the optical spectra of such samples are consistent with complete reduction of both hemes (data not shown). It should be noted that the reduction of the modified copper is reversible: reexposure of a di-

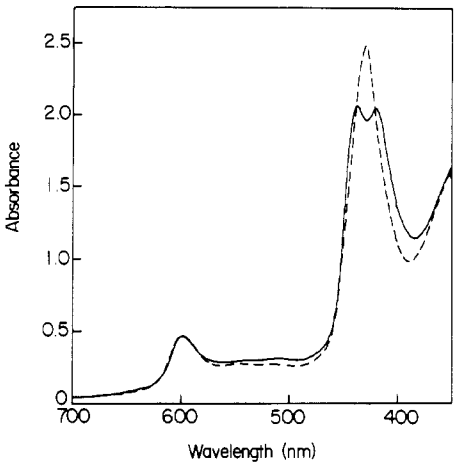

FIGURE 5: Reduction of the hemes of pHMB-modified cytochrome oxidase with NADH and PMS. Optical spectra of an anaerobic solution of pHMB-modified cytochrome oxidase. Enzyme concentration approximately 110 μM. (Solid trace) After reduction with 430 μM NADH and 0.93 μM PMS at 0 °C for 75 min. (Dashed trace) Same sample after reduction for 90 min and equilibration with 1 atm of CO.

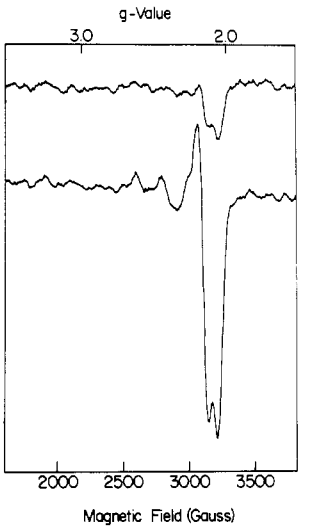

FIGURE 6: Reduction of the EPR-visible copper centers in native and pHMB-modified cytochrome oxidase by NADH and PMS. EPR spectra of anaerobic enzyme samples incubated with NADH and PMS under the conditions of Figure 5A. (Top trace) Cytochrome oxidase (unmodified control). (Bottom trace) pHMB-modified cytochrome oxidase. Conditions for EPR spectroscopy same as for Figure 3A.

thionite-reduced sample of the modified enzyme to air results in the reappearance of Fe_a^{3+} and modified copper EPR signals identical with the original oxidized signals (Figure 4C). These results provide strong evidence for the existence of a competent electron-transfer pathway in the pHMB-modified enzyme which directly or indirectly connects both the EPR-visible copper and the Fe_a centers to the oxygen-binding site.

Reduction of the pHMB-Modified Enzyme by Ascorbate/Cytochrome c or NADH/PMS and Reoxidation by O_2. Sodium dithionite is thought to reduce cytochrome oxidase by transferring electrons into the enzyme primarily through the oxygen-binding site rather than through the physiologically relevant reduction pathway involving the cytochrome *c* binding site (Halaka et al., 1981). In contrast, cytochrome *c* (reduced by sodium ascorbate, which does not reduce cytochrome oxidase directly) and phenazine methosulfate (reduced by NADH, which does not reduce cytochrome oxidase directly) are both known to react with the enzyme *via* the cytochrome *c* binding site (Antalis & Palmer, 1982; Halaka et al., 1981). These reductants can therefore be used in experiments to examine the functioning of this electron-transfer pathway. Under steady-state aerobic conditions, electron transfer to all of the metal centers in native cytochrome oxidase is rather rapid (turnover > 10^2 s^{-1}). When cytochrome oxidase is treated with NADH/PMS or ferrocytochrome *c* in the absence of O_2, however, Fe_a^{3+} is still reduced rapidly but $Fe_{a_3}^{3+}$ reacts at a much slower rate (Antalis & Palmer, 1982).

The hemes in the pHMB-modified enzyme exhibit similar behavior to those in the native enzyme when the modified protein is treated with reductants that act at the cytochrome *c* binding site. After reduction with NADH/PMS for 75 min, the optical spectrum (Figure 5) of the enzyme shows two Soret peaks (438 and 421 nm), one of which displays the shift to longer wavelengths expected from reduction of a heme chromophore. EPR spectra of this sample revealed no detectable Fe_a^{3+} signal at $g = 3.0$, confirming the reduction of this center to Fe_a^{2+}. Incubation of the sample for longer times resulted in a progressive intensity increase of the longer wavelength optical band and concomitant diminution of the shorter wavelength band, as is expected if the initial reduction of Fe_a is followed by the slower reduction of Fe_{a_3}; this process is somewhat slower in the modified enzyme than in the unmodified control. [It should be noted, however, that the rates of electron transfer to the oxygen-binding site under anaerobic conditions are not necessarily relevant to kinetics under turnover (i.e., aerobic) conditions. It is thought that the effective reduction potential of this site is greatly increased in the presence of O_2; this change in reduction potential is associated with a dramatic increase in the rate of electron transfer to the oxygen-binding site from Fe_a^{2+} and Cu_A^+ (Antalis & Palmer, 1982).] Finally, addition of CO to a sample which had been reduced with PMS/NADH for 90 min immediately gave rise to the dashed spectrum in Figure 5; identical spectra were obtained even when the CO was added after an extremely long (ca. 50 h) incubation with NADH/PMS (data not shown). Since CO binds only to the reduced Fe_{a_3} heme, the CO-induced spectral change must involve production of a species containing an Fe_{a3}^{2+}–CO complex. In fact, the observed spectrum (λ_{max} 429.5) is almost identical with that of the fully reduced CO complex of native cytochrome oxidase (λ_{max} 430.0), and EPR spectroscopy at 10 K confirms that Fe_a is reduced in this species.

Similar results were obtained when an ascorbate/cytochrome *c* mixture was employed as the reductant. When O_2 was introduced to the reduced or partially reduced enzyme samples, reoxidation of both the cytochrome oxidase and the excess ferrocytochrome *c* reductant was observed, again demonstrating that the modified oxidase has the ability to catalyze the oxidation of cytochrome *c* by O_2. The optical absorbance change at 442 nm associated with the reoxidation of both the modified and unmodified control samples is completed on a time scale faster than that observable by manual mixing methods (ca. 10 s). Taken together, the results of the single-turnover experiments indicate that the iron centers of the modified enzyme retain the electron-transfer functions of those in the native enzyme.

In contrast to the redox behavior of the heme irons, the response of the EPR-visible copper to the milder reductants is inhibited after reaction of the enzyme with pHMB. The top spectrum in Figure 6 demonstrates that, in the native enzyme, both Fe_a and Cu_A are largely reduced by treatment with NADH and PMS under anaerobic conditions. When the pHMB-modified enzyme is incubated under identical conditions, Fe_a is again reduced, but the EPR signal from the modified copper center is still present, indicating that this site

remains in its cupric oxidation state. Even when a sample of the modified enzyme is treated with NADH/PMS for as long as 50 h, a decrease in the intensity of the modified Cu^{2+} EPR signal is not observed. The optical spectrum of this sample is consistent with complete reduction of both hemes (data not shown). Since the EPR spectra in Figure 6 are taken at cryogenic temperatures, the positions of the redox equilibria between the Cu^{2+} and potential reductants studied under these conditions are not necessarily the same as those at physiological temperatures. We have therefore examined the redox properties of the modified copper center by exposing it to the physiological reductant, cytochrome *c* (reduced by the addition of excess sodium ascorbate), and recording the copper EPR signal intensity at a substantially higher temperature (−20 °C). Comparison of the mean relative intensity of the modified Cu_A^{2+} signals in the samples after the addtion of reductant with that before reduction and after reoxidation with air (6.6 ± 1.4 and 6.0 ± 1.0, respectively) reveals no reduction of the modified copper under these conditions. Thus, the pHMB-modified copper center is not substantially reduced under conditions of applied reduction potential similar to those under which the substantial steady-state turnover activities were measured.

Discussion

It is unlikely that the chemical modification of cytochrome *c* oxidase by pHMB described in this paper is a *specific* chemical modification of cytsteine ligand(s) of Cu_A. Tsudzuki et al. (1967) treated cytochrome oxidase with pCMB and demonstrated that approximately 12 of the 14 mol of thiol groups present in 1 mol of the beef heart enzyme was modified. Since we have employed both a much higher concentration (of pHMB) and a much longer reaction time for the preparation of the pHMB-modified enzyme, we expect that this reagent has also reacted with a majority of (or possibly with all of) the thiol groups present. It is therefore perhaps somewhat surprising that the reaction with pHMB does not cause more global changes in the structural and functional properties of the metal center active sites of the enzyme. The spectroscopic studies of the pHMB-modified enzyme do nonetheless demonstrate that, despite the major changes to the EPR and optical signatures of Cu_A, the EPR signals arising from the other oxidase metal centers are identical with the corresponding signals from the native enzyme. It is important to note that the EPR *g* values and hyperfine coupling constants of a transition metal ion depend in a sensitive way on the electronic structure of the ion and are therefore responsive to relatively minor changes in ligation. In the cases of ferric heme (Peisach & Blumberg, 1974) and of Cu^{2+} (Peisach, 1978) sites with ligands of the types found in metalloproteins, changes in the identity of ligands will almost invariably cause large, easily observable changes in the X-band EPR spectrum.

The optical absorption features of the cytochrome *c* oxidase hemes are also essentially unchanged by the modification; this observation is also consistent with the absence of major structural changes at these sites. In the absence of changes in the EPR spectra, the observed slight shifts in the peak positions of the heme optical bands most likely arise from small changes in the environment of the porphyrin macrocycle rather than from changes in the coordination environment of the iron. Even a change in the hydrogen bonding of a substituent on the periphery of the heme *a* ring can cause the type of perturbation of the optical spectrum noted here (Callahan & Babcock, 1983).

Structure of the Modified Copper Center. Comparison of the spectroscopic properties of the native and pHMB-modified enzyme preparations shows clearly that treatment of the enzyme with pHMB induces a structural change at the Cu_A site; however, there is insufficient information to unambiguously determine the structure of the modified copper center and the nature of the chemical transformations which produce it. The most straightforward mechanistic interpretation of the data is that the pHMB reacts with one or more cysteinate ligand(s) of Cu_A, thereby weakening or eliminating entirely the association of said ligand(s) with the copper ion and thus causing the changes in the electronic structure of the site which manifest themselves in the EPR and optical spectra. The assignment of the hyperfine structure in the g_y region of the EPR spectrum of the modified copper center to coupling with *three* equivalent ^{14}N nuclei is consistent with at least one ligand substitution at the site, since there are thought to be no more than two nitrogenous ligands to Cu_A in the native enzyme (Stevens et al., 1982; Martin, 1985; C. T. Martin, C. P. Scholes, and S. I. Chan, unpublished results). It is also possible, however, that the reaction with pHMB of cysteine residues more distant from the Cu_A site induces conformational changes in the protein. If this is the case, these reactions might indirectly cause an alteration of the ligation structure at the Cu_A site, thereby inducing the spectroscopic changes.

Although the available data do not allow an unambiguous determination of the chemical nature of the modified copper center, several of the experiments demonstrate that the modified enzyme molecules retain their full complement of structurally intact Cu_B sites. This modified copper center must therefore be derived from Cu_A only. Observations which support this conclusion are the following: (1) The total intensity of the Cu^{2+} EPR signal from the modified enzyme corresponds to 1 mol of Cu^{2+}/mol of enzyme. This observation is inconsistent with the production of an EPR-visible species from the (normally EPR invisible) Cu_B center. (2) Production of an EPR-visible species from Cu_B^{2+} would require the elimination of the strong exchange coupling of this site with $Fe_{a_3}^{3+}$, and the latter center would then also be expected to exhibit an EPR signal; however, no substantial amounts of new signals that could be attributed to Fe_{a_3}-derived species are observed in the EPR spectrum of the pHMB-treated enzyme (Figure 3A). (3) The production of the cyanocytochrome a_3 EPR signal requires that CN^- and NO bind in a specific arrangement to both $Fe_{a_3}^{3+}$ and Cu_B^{2+} (Chan et al., 1980; Brudvig et al., 1981). The observation that essentially all of the modified enzyme molecules can be converted into this form (Fig. 3B) is strong evidence that they contain functionally intact oxygen binding sites in which *both* metal centers display the same chemical properties as their analogues in the native enzyme. (4) The rapid four-electron reduction of oxygen by cytochrome oxidase is thought to involve ligation of both Fe_{a_3} and Cu_B by partially reduced intermediates derived from O_2 (Blair et al., 1983; Malmström, 1983). The substantial turnover rate of the modified enzyme thus suggests that this site is functionally intact.

Redox Reactions of the Modified Enzyme. The absence of pHMB-induced changes at the metal center sites other than Cu_A is reflected in the behavior of the modified enzyme in its reactions with reductants and with O_2. Like those in the native enzyme, all four of the metal center sites in the pHMB-modified oxidase are reduced by sodium dithionite and can subsequently be rapidly reoxidized by O_2. It should be noted that the lack of inhibition observed in the single-turnover experiments is consistent with the observations of Tsudzuki et al. (1967), who noted essentially no decrease in the rate of turnover of cytochrome oxidase when the enzyme was modified

by pCMB. As previously discussed, the reaction conditions we have used for the pHMB modification probably cause modification of a larger number of sulfhydryl groups than occurred in the earlier work. This may account for the greater inhibition of the turnover kinetics which we observe; however, it is also possible that some of the inhibition is due to chemical effects of the pHMB other than local modification of the Cu_A site.

The most striking difference between the chemical properties of the native and pHMB-modified enzyme preparations involves their reactions with reductants. The EPR-visible Cu^{2+} centers of both preparations are rapidly and completely reduced by sodium dithionite. Unlike Cu_A in the native enzyme, however, there is no detectable reduction of the pHMB-modified copper center when the enzyme containing it is treated with either ascorbate/cytochrome *c* or NADH/PMS. One possible explanation for this effect of the pHMB modification is a change in the thermodynamic properties of the site. Specifically, the modification might lower the reduction potential of the copper center to such an extent that it can no longer be significantly reduced by ascorbate or NADH, but can be reduced when the much more powerful reductant sodium dithionite is used under otherwise identical conditions. This would be the case if the standard reduction potential of the modified copper center falls in or near the range delimited by the potentials of dithionite [$E^{0'} = -0.527$ V (Loach, 1976)] and of NADH [$E^{0'} = -0.320$ V (Loach, 1976)]. This range of potentials is substantially lower than that typical of copper proteins (Addison, 1983; Brill et al., 1964) but is in the region in which the potentials of many small organocopper complexes occur (Patterson & Holm, 1975). An alternative explanation for the failure of both ascorbate/cytochrome *c* and NADH/PMS to reduce the modified copper center is that the electron transfer from these reductants to the copper center is significantly slower in the modified enzyme but that electron transfer from dithionite is not substantially affected. Although such a change in the kinetic properties of the enzyme cannot be entirely discounted, it seems unlikely that it would be sufficient to explain the observed effects of pHMB modification on the redox processes. In this context, the experimental observation that no reduction of the modified copper EPR signal intensity occurs even after 50 h of treatment with NADH/PMS is particularly germane. If production of reduced copper is actually taking place under these circumstances, it can only be doing so at a rate many orders of magnitude slower than that in the native enzyme (Antalis & Palmer, 1982).

Catalytic Turnover of the pHMB-Modified Enzyme. In the nearly 20 years since Gibson & Greenwood (1965) demonstrated that the redox state of Cu_A can change as rapidly as that of Fe_a in response to reaction of the enzyme with reducing or oxidizing substrates, the importance of this center in cytochrome oxidase electron-transfer reactions has become increasingly well established. In the cytochrome oxidase derivative descibed in this paper, the Cu_A center has been converted into a form which is no longer redox active with the enzyme's substrate, ferrocytochrome *c*. It is therefore not surprising that the preparation of this derivative is accompanied by the loss of the *majority* of the catalytic cytochrome oxidase activity present in the native enzyme. As described under Results, however, the oxygen consumption measurements demonstrate the presence of a significant residual turnover activity in the modified enzyme which amounts to approximately 20% of that of the native enzyme. Since the modified copper center is not significantly reduced by ascorbate/cytochrome *c*, it is unlikely that this center can catalyze intramolecular electron-transfer reactions at a significant rate under the conditions of the activity measurements, in which ascorbate is the strongest reductant present. For a modified copper center with a standard reduction potential more than 0.5 V below those of Fe_a and cytochrome *c*, virtually no net flux of electrons through the site is expected, even if the rate of reduction of the oxygen binding site by the copper is substantially increased because of the increase in the free energy change associated with this reaction. [The effect on the reaction rate of changing the potential of the electron-transfer catalyst is analogous to that encountered when mediators of various potentials are used to catalyze electron transfer between solution species and electrodes; this phenomenon is discussed by Clark (1960).] The presence of residual turnover activity must therefore reflect the presence in the modified enzyme of a Cu_A-independent electron-transfer pathway from cytochrome *c* to Fe_a and to the oxygen-binding site. In view of the fact that the modified enzyme appears to be largely unperturbed by the modification (with the exception of the changes at Cu_A), it is probable that such a pathway exists in the native enzyme as well. These observations therefore strongly suggest that *direct* electron transfer (without the mediation of Cu_A) between cytochrome *c* and Fe_a can occur at a rate which is at least as large as one-fifth of the overall turnover rate of the unmodified enzyme.

The existence in the native enzyme of a branched electron-transfer pathway, having the major flow of electrons occurring from Cu_A to the oxygen-binding site and a minor flow from Fe_a, was proposed by Clore et al. (1980) as the model which best fit the data from their careful EPR and optical kinetics studies of the low-temperature intermediates in the reaction of reduced cytochrome oxidase with O_2. Recent studies using a similar technique in our laboratory have confirmed that a linear (unbranched) pathway is insufficient to explain the observed kinetics and have extended the observations into later stages of the electron-transfer cycle and over a broader range of temperatures. As in the study described above, electron transfer from Cu_A^+ to the oxygen-binding site was observed to be the dominant pathway with a smaller but significant (ca. 30% of the total) amount of transfer occurring from Fe_a^{2+}. No significant change in the ratio of the rates of the two pathways was observed over a substantial temperature range (170–195 K) (D. F. Blair, S. N. Witt, and S. I. Chan, unpublished results).

The observation of electron-transfer kinetics consistent with a minor Cu_A-independent electron-transfer pathway together with the direct demonstration of such a pathway in the pHMB-modified enzyme creates a strong case that direct Fe_a^{3+} to oxygen-binding site electron transfers can in fact occur in cytochrome *c* oxidase in respiring mitochondria. Although it is possible that such transfers merely represent a small "leakage" pathway which is of little physiological significance, we favor an alternative interpretation. The electrochemical hydrogen ion gradient which is generated by cytochrome oxidase turnover is produced by two distinct mechanisms: by consumption of H^+ from the matrix of the mitochondrion during the conversion of O_2 to H_2O, and by proton pumping, in which the large amounts of energy liberated under physiological conditions by the reduction of O_2 by cytochrome *c* are used to fuel the (energetically unfavorable) transfer of H^+ ions from the matrix side of the mitochondrial inner membrane to the cytosolic side. We have proposed, on the basis of a wide variety of experimental data about the structures and reactions of the metal center sites, that the cytochrome oxidase proton

pump is localized at the Cu_A site and is driven by electron transfer through this site (Chan et al., 1979). If this were the case, the presence of a minor electron-transfer pathway which bypasses Cu_A would cause a small decrease in the efficiency of coupling; however, the existence of such a pathway would confer the advantage of allowing catalytic turnover of the enzyme under a much wider range of physiological conditions. This is because the change in free energy associated with the *overall* cytochrome *c* oxidase reaction (electron transfer, oxygen reduction, and proton pumping) must become less negative as the energization of the membrane increases; if the electrochemical proton gradient becomes too large, the free energy change will become zero and the reaction will no longer proceed spontaneously. If an electron-transfer pathway which bypasses the proton pump is present, however, turnover under this condition and even under those of somewhat higher energization will be possible because an energy-requiring step (proton pumping) is eliminated from the catalytic cycle. Thus, the bypass pathway which only a small fraction of the electrons use under nonenergized conditions would become dominant under highly energized conditions. The fraction of the total electron-transfer rate occurring through each of the two pathways would also vary if some of the four successive electron transfers to the O_2-binding site which are necessary to reduce O_2 to H_2O produce more energy than do others (Malmström, 1982). In both cases, such an arrangement allows the enzyme to tailor its coupling efficiency to changes in the relationship between the amount of energy available from electron-transfer reactions and the amount needed to pump protons. This type of compromise between energy efficiency and turnover rate may be necessary for the efficient overall functioning of the metabolic pathway.

The reaction between pHMB and cytochrome oxidase under the conditions described in this paper represents the first reported chemical modification of the Cu_A site which is not accompanied by extensive denaturation and inactivation of the protein. We anticipate that this derivative of the enzyme will prove to be a valuable tool in future studies of the role that Cu_A plays in the catalytic mechanism of cytochrome oxidase.

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Registry No. pHMB, 138-85-2; Cu, 7440-50-8; cytochrome *c* oxidase, 9001-16-5.

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Effect of Dicyclohexylcarbodiimide on Unisite and Multisite Catalytic Activities of the Adenosinetriphosphatase of *Escherichia coli*†

Massimo Tommasino and Roderick A. Capaldi*
Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403
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ABSTRACT: The inhibitory effect of dicyclohexylcarbodiimide (DCCD) on the activity of the adenosinetriphosphatase of *Escherichia coli* (ECF_1) has been examined in detail. DCCD reacted with ECF_1 predominantly in β subunits with a maximum of 2 mol of reagent per mole of ECF_1 being incorporated in these subunits. Ninety-five percent inhibition of steady-state or multistate ATPase activity required incorporation of 1 mol of DCCD per mole of enzyme into β subunits. Seventy-five percent inhibition of the initial rate of unisite catalysis was only obtained after incorporation of 2 mol of DCCD per mole of ECF_1 into β subunits. Analyses of the kinetics of unisite catalysis and nucleotide binding experiments both indicate that DCCD binds outside the substrate ATP binding site. Inhibition by this reagent appears to be due in part to an effect on the catalytic sites but mainly to the blocking of cooperativity between these sites.

F_1 ATPases from bacteria, plant chloroplasts, and animal and plant mitochondria are similar complexes of M_r 350 000-380 000 made up of five different subunits, α, β, γ, δ, and ϵ, now generally agreed to be present in the stoichiometry of 3:3:1:1:1, respectively (Futai & Kanazawa, 1983; Senior & Wise, 1983; Fillingame, 1980). The active site for both ATP synthesis and ATP hydrolysis in the enzyme is thought to be located on β subunits with a possible contribution from the α subunits (Futai & Kanazawa, 1983; Senior & Wise, 1983). On the basis of considerations of stoichiometry, therefore, there should be three catalytic sites per complex. Direct evidence for at least two and probably three sites of ATP hydrolysis has been obtained by substrate binding experiments and from kinetic studies of the ATPase activity of the enzyme (Cross & Nalin, 1982; Nalin & Cross, 1982; Grubmeyer et al., 1982; Grubmeyer & Penefsky, 1981a,b).

F_1 ATPase from all of the sources listed above show dramatic cooperativity between active sites, and this is reflected in the rate of ATP hydrolysis under different conditions of enzyme to substrate concentration ratios. Thus, the rate of ATP hydrolysis is very slow when there is more enzyme than substrate, the rate-limiting step of this single-site or unisite catalysis being the slow off rate of product ADP (Grubmeyer & Penefsky, 1981b; Grubmeyer et al., 1982; Gresser et al., 1982). When there is an excess of substrate to enzyme, i.e., under steady-state or multisite assay conditions, the rate of ATPase activity is much faster, i.e., by a factor of 10^4-10^6 over that in unisite catalysis (Grubmeyer et al., 1982). The much enhanced rate of catalysis when two or more active sites per

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