Structural Perturbation of the a_3–Cu_B Site in Mitochondrial Cytochrome *c* Oxidase by Alcohol Solvents†

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ABSTRACT: Ethanol has been observed to cause a perturbation of the catalytic center of the major respiratory protein cytochrome *c* oxidase. These effects were examined by Fourier transform infrared spectroscopy of carbon monoxide complexes of cytochrome a_3Fe and of Cu_B formed by low-temperature photodissociation of the a_3FeCO complex. Carbon monoxide binds to reduced cytochrome oxidase in two major structural forms, α and β, both of which are altered by ethanol. In the absence of ethanol, 15–22% of the total cytochrome oxidase in beef heart mitochondria was observed as β-forms. Ethanol addition caused a concentration-dependent elimination of the β-forms with 40% disappearing at 0.05 M (0.23%) ethanol, a concentration that can readily be achieved in the blood of intoxicated individuals. At 0.5 M (2.3%) ethanol and above, almost no β-forms were detectable. The α-Cu_BCO absorption normally splits into two bands at temperatures below 40 K. This effect was decreased in the presence of ethanol and eliminated by high ethanol concentrations. It appears that ethanol increases the structural fluctuations at the active site of the enzyme, analogous to the effects of increased temperature. There was an 8–10% decrease in the maximum rate of oxygen reduction by mitochondrial cytochrome oxidase in 0.05 M ethanol at 24 °C, while higher concentrations of ethanol caused no further inhibition. This is the first demonstration that α- and β-forms of cytochrome *c* oxidase can be modified by an externally added reagent. Changes in the spectra of α-Cu_BCO in the presence of 50% (v/v) ethylene glycol were quite striking, but variable. In contrast, the effect of glycerol appears to be limited to extraction of water, as samples prepared with glycerol had spectra similar to those of aqueous preparations. Low-temperature splitting of the α-Cu_BCO band is consistent with an exchange process due to H-bond formation between an ionizable ligand to α-Cu_B and an adjacent ionizable group from the protein. Ethanol disrupts this interaction. A model is presented. We suggest that these data require the presence of a mobile proton adjacent to the coordinated Cu_B complex. Such a proton may participate in dioxygen reduction to water or in proton pumping mechanisms. A corresponding exchange process is not observed in β-forms of cytochrome *c* oxidase.

We have previously shown that heart mitochondrial cytochrome *c* oxidase can exist in several structures that are defined as α- and multiple β-forms (Alben et al., 1981; Fiamingo et al., 1982) and that the relative amounts of these structural forms depend upon the preparation (Fiamingo et al., 1986, 1988). With the use of Fourier transform infrared spectroscopy of the reduced enzyme exposed to carbon monoxide, these structural forms can be described and quantitatively distinguished by the absorption characteristics of the CO bound at the a_3Fe–Cu_B catalytic site. Photodissociation of a_3FeCO produces a Cu_BCO complex that is stable below 140 K but dissociates to re-form the a_3FeCO complex at higher temperatures. We have observed two types of cytochrome oxidase a_3FeCO structures: an α-form, characterized by a narrow absorption band (2.4–3.2-cm^{-1} bandwidth) centered at 1963 cm^{-1} and observed in every sample studied, and several β-forms with broader (3.5–15-cm^{-1} bandwidth), less intense absorptions between 1947 and 1977 cm^{-1}. The α-FeCO band photodissociates to an α-CuCO band centered at 2062 cm^{-1} that splits into two bands below 40 K, while the β-FeCO bands all appear to photodissociate to a single β-CuCO band at 2043 cm^{-1} that does not split at lower temperature. The fraction of the FeCO band area belonging to the β-forms has been observed at 46% in opossum heart tissue (Fiamingo et al., 1988), 44–51% in isolated rat heart myocytes, which decreased to 21% after digitonin treatment lysed the sarcolemma while leaving the inner mitochondrial membrane intact (Fiamingo et al., 1986), and 0–27% in isolated beef heart mitochondria (Fiamingo et al., 1982). This suggested that *cytochrome oxidase structure may be modified by soluble components that interact either directly with the enzyme or indirectly through changes in the properties of the mitochondrial membrane.*

In this report we show that the concentration of β-forms of cytochrome *c* oxidase and the α-Cu_B structure, as measured by the degree of splitting at low temperature, are both greatly affected in a very specific manner by the presence of ethanol at low concentration or less specifically by ethylene glycol at high concentration. Ethanol also slightly decreases the maximal rate of oxygen reduction by cytochrome oxidase. In contrast, glycerol appears to preserve the structure at the metal centers found in aqueous samples. In addition, we shall relate the solvent effects of these alcohols to the importance of various forms of cytochrome *c* oxidase for respiratory control and the possible role of the Cu_B site as a proton donor during the

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catalytic reduction of dioxygen.

Materials and Methods

Beef heart mitochondria were prepared by using Nagarse (Jung et al., 1977) and suspended (25 mg of protein/mL) in sucrose (0.25 M) containing Tris buffer (2 mM, pH 7.4). For some of the infrared studies the mitochondria were diluted by the addition of concentrated sucrose solution to 20 mg of protein/mL in 0.4 M sucrose. For studies of alcohol effects, ethanol was added to these suspensions to final concentrations of 0.005–1.5 M (0.023–6.9 wt %). Alternatively, ethylene glycol was added to the aqueous mitochondrial suspension to bring the solution to 50% ethylene glycol (v/v). For the infrared studies the mitochondria were then centrifuged at 12000*g*. The resulting aqueous pellet was pressed between a pair of CaF_2 windows with a 0.095-mm optical path length and mounted in a cryostat.

Infrared spectra were obtained with a Mattson Sirius 100 Fourier transform infrared interferometer at 0.5-cm^{-1} resolution. A liquid nitrogen cooled indium antimonide detector was used to observe the spectra in the 1750–3000-cm^{-1} range, which permitted observation of both the iron-bound and photodissociated CO. Interferograms were recorded in both directions of mirror movement through a 16-bit A/D converter and signal-averaged (2048 scans in each direction) into 32-bit words, which were used for all further computations. Full double-sided interferograms were triangularly apodized, with the real part of their Fourier transforms resulting in the single-beam spectra. Low-temperature spectra were compared with spectra from the same sample after photodissociation with a 500-W tungsten lamp focused through a slide projector and optically filtered through glass and water to remove ultraviolet and infrared radiation. Subtraction of least-squares fits of a cubic polynomial to the base-line regions of the spectra was used for base-line correction. There was no further averaging, smoothing, or other correction of the spectra. Band areas were measured by computer integration of the base-line-corrected spectra.

Low temperatures were maintained and measured with a Lake Shore Cryotronics helium refrigerator (Model LTS-21-D70C) and digital thermometer (Model DRC-70) with a calibrated silicon diode probe (±0.5 K).

Respiration was measured by using a YSI oxygen electrode with a temperature-regulated cell at 24 °C. Beef heart mitochondria were suspended (0.88 mg of protein/mL) in a medium of KCl (100 mM), TES [*N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid] (10 mM, pH 7.1), EGTA [[ethylenebis(oxyethylenenitrilo)]tetraacetic acid] (30 μM), potassium phosphate (3 mM), ascorbate (5 mM), and TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) (50 μM) in a final volume of 5.7 mL.

Results

Ethanol Effects on Cytochrome c Oxidase Structure. Ethanol in concentrations of 0.005–1.5 M (0.023–6.9%) produced a loss of β-FeCO and β-CuCO infrared absorption bands with increasing concentration, with half-maximal effect at 0.05–0.15 M ethanol (0.23–0.69%) and nearly complete loss at 0.5 M ethanol (see Figure 1). At the same concentration, changes were also observed in the properties of the α-form. At 10 K the α-FeCO absorption shifted to higher frequency and the bandwidth broadened with increasing ethanol concentration, as illustrated in the difference spectra in Figure 2A. The α-CuCO band showed a progressive decrease in the degree of splitting, with only a single band evident at concentrations of 0.5 M ethanol and above. The control samples (no ethanol) clearly showed a normal split α-CuCO band at 10 K that is not observed in the 0.5 M ethanol sample (Figure 1C). The elimination of the β-forms (Figures 1 and 2) and decreased splitting of the α-CuCO band by ethanol were concentration dependent.

With increasing ethanol concentration the α-CuCO spectra were altered in a manner consistent with the increased structural disorder observed at low temperature, e.g., 10 K (Figures 1C and 2B). This is analogous to raising the temperature of the control sample, as shown in Figure 3. The characteristics of the α-CuCO splitting are consistent with an exchange-type process, whereby the system alternates between two distinct states. As the temperature increases, the observed frequency is an average of these two, weighted by the time spent in either state. Above 60 K, even in the absence of ethanol, little structure is observed in the α-CuCO band shape.

A further indication of local protein structural disorder (associated with fluidity at higher temperatures) can be seen by the increased bandwidth of the α-FeCO resonance. The increased bandwidth causes a decreased absorptivity at the band maximum that can best be illustrated by comparing the CuCO and FeCO band amplitudes at 160 K (a temperature at which there is no observed CuCO splitting), as shown in Figure 4. There is a monotonic increase in the amplitude ratio versus ethanol concentration in the sample, with an increase of 77% at 1.5 M ethanol. The data can be approximated by the function

$$y = A(1 + e^{-x/X\ln 2}) + B$$

where X is the value of x at 50% effect [0.22 M ethanol (1.0%)], x is the ethanol concentration (M), and y is the amplitude ratio of CuCO/FeCO.

The reversibility of these effects was examined. After mitochondria in 1.5 M ethanol were spun down, the supernatant was removed and the pellet was resuspended in ethanol-free CO-saturated buffer. In the pellet from this sample we observed a small degree of α-CuCO splitting at 10 K and a decreased CuCO/FeCO amplitude ratio (shown as the open square in Figure 4). These spectral parameters were similar to those of a parallel sample at 0.15 M ethanol. Allowing for the retention of water by the original mitochondrial pellet, this was close to the expected concentration of ethanol in the resuspended sample. We have not yet observed a reversibility of the ethanol effects on the β-forms of cytochrome oxidase.

Ethylene Glycol Effects on Cytochrome c Oxidase Structure. Ethylene glycol at 50% (v/v) produced dramatic but variable changes in the cytochrome oxidase spectra, especially at the Cu_BCO site (Figure 5). One sample at 10 K exhibited a β-Cu_BCO band at 2038 cm^{-1} with a strongly split α-Cu_BCO absorption (each with about equal intensity) at 2055 and 2062 cm^{-1}. The β-CuCO signal changed sharply with temperature, moving to 2048 cm^{-1} at 160 K, while at this temperature the α-CuCO signal is a singlet at 2061 cm^{-1} (not shown). The lower frequency band of the α-CuCO doublet decreased in intensity as the temperature increased, while the higher frequency component had very little change with temperature. A second sample at 15 K exhibited no splitting of the α-CuCO absorption at 2061 cm^{-1}, except for a small shoulder at 2046 cm^{-1} (Figure 5). This shoulder was probably due to a β-CuCO structure of the enzyme, since it had the expected intensity to correspond with the β-FeCO absorption.

Similarity of Cytochrome c Oxidase Spectra in Water and Glycerol. In contrast to the effects of ethylene glycol, aqueous samples (Figure 3) and samples dehydrated by overlaying the mitochondrial pellet with CO-saturated glycerol (Fiamingo et al., 1982) have a β-CuCO absorption, 2039 cm^{-1} at 10 K,

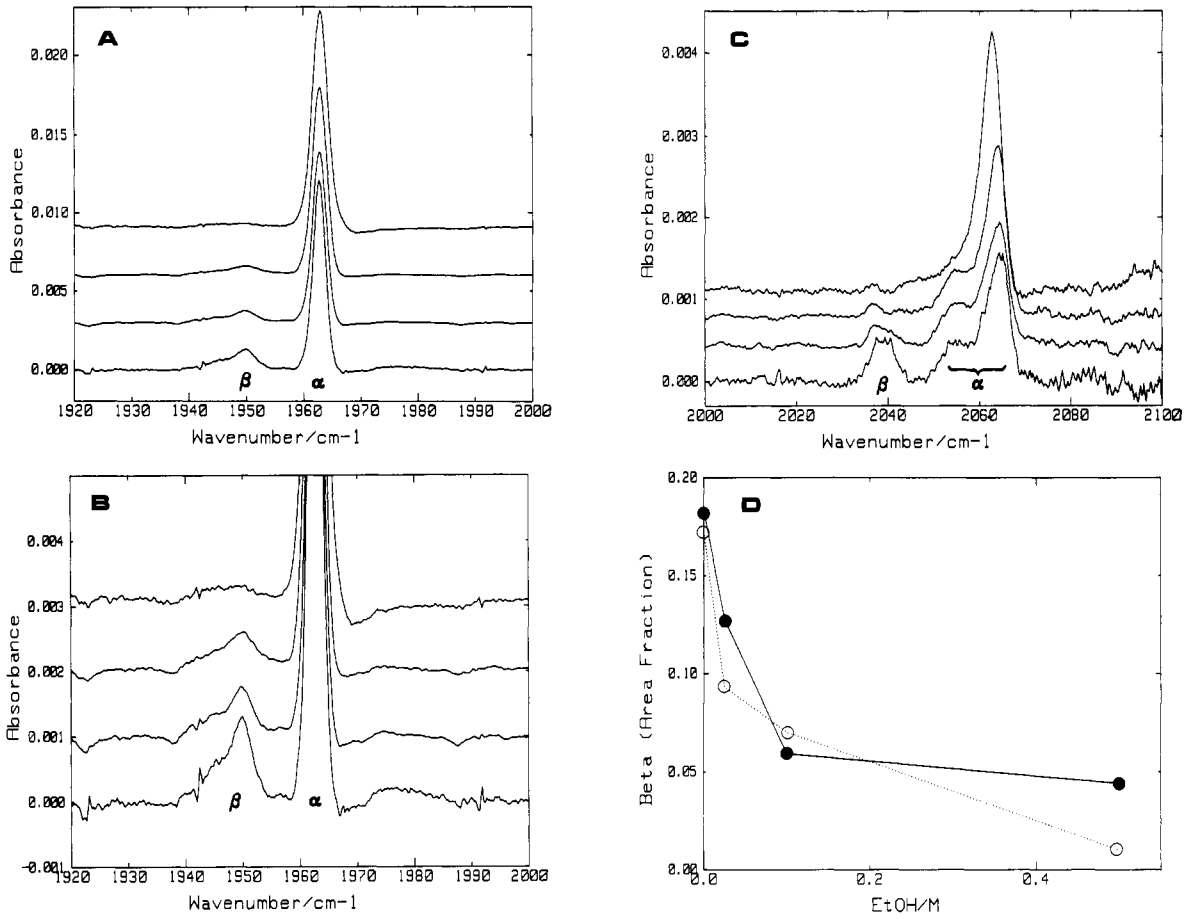

FIGURE 1: Infrared absorbance difference spectra (light minus dark) of cytochrome oxidase from beef heart mitochondria at 10 K. The bottom spectrum was from a sample not exposed to ethanol; the spectra above this were exposed to 0.025, 0.1, and 0.5 M ethanol, respectively. (A and B) a_3FeCO: The β-forms are observed near 1950 cm^{-1} and the α-form is centered at 1962.7 cm^{-1}. (C) Cu_BCO: The β-forms are centered at 2039 cm^{-1} and the α-form is observed at 2063 cm^{-1} (0.5 M ethanol) and at 2054 and 2065 cm^{-1} (α-form, no ethanol). The spectra were normalized to the same total FeCO area. (D) Fraction of total band area observed as β-forms of cytochrome *c* oxidase for FeCO (—) and CuCO (···) versus ethanol concentration.

that shifts to 2043 cm^{-1} at 160 K. They have an α-CuCO doublet at 2055 and 2065 cm^{-1} at 10 K, where the lower frequency component is about one-third the intensity of the other, and both bands move toward 2062 cm^{-1} as the temperature is raised. The effect of glycerol appears to be limited to the extraction of water from the membrane–protein matrix, and glycerol probably does not penetrate the membrane.

Ethanol Effect on Mitochondrial Respiration. In order to examine whether the changes in cytochrome oxidase structure that we observe at very low temperatures are important for enzyme function at higher temperature, the respiration rates of mitochondria at 24 °C were measured. TMPD plus ascorbate was used as substrate to feed electrons to cytochrome *c*. Ethanol has very different effects on respiration stimulated by ADP [state 3, Chance and Williams (1956)] or by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Figure 6). State 3 respiration decreased in direct relation to increasing ethanol concentration with no ADP stimulation of respiration occurring at 1.5 M ethanol. In contrast, CCCP-stimulated respiration was reduced 8–10% at 50 mM ethanol with no further inhibition observed at higher ethanol concentrations. Nonstimulated respiration rates [state 4 and the initial rate before ADP addition (Figure 6A), as well as the rate before CCCP addition (Figure 6B)] all show a 9–13% decrease at 50 mM ethanol, with respiration being stimulated at higher ethanol levels. At 1.5 M ethanol, state 4 was increased 23% and became equal to state 3, resulting in loss of respiratory control. The increase in state 4 may result from a stimulation of ATPase activity, as it becomes apparent only after ATP becomes available.

Uncoupler-stimulated respiration is the most direct measurement of cytochrome oxidase activity, since it is only dependent on uncoupler effectiveness in shuttling protons across the membrane and collapsing the proton electrochemical potential. The effect of ethanol on cytochrome oxidase at 24 °C appears to be limited to a 10% reduction in activity at 50 mM ethanol. This may correspond to loss of β-cytochrome oxidase or to modification of the Cu_B site of the α-form to convert it to a less active structure.

Discussion

Solvent affects cytochrome *c* oxidase in a complex fashion. For the purpose of discussion, these effects may be divided into two mechanisms. First is a general disordering of the mitochondrial membrane associated with the absorption of ethanol, and second is a specific binding of ethanol at the metal center. We must also distinguish α- and β-forms of cytochrome *c* oxidase and focus on structural interactions of the α-Cu_BCO site and its surroundings. The frequency difference of the carbonyl absorption, as well as differences in kinetic behavior, suggests a different coordination sphere for the α-CuCO from that found in the β-form.

The spectral difference (23 cm^{-1}) between the α-form (2063 cm^{-1}) and β-form (2040 cm^{-1}) of Cu_BCO reflects differences in the local molecular environment of the two forms. This could possibly be due either to H-bonding directly to the β-CuCO or to a steric effect that forces the β-CuCO to have a more acute angle than the α-CuCO or to differences in

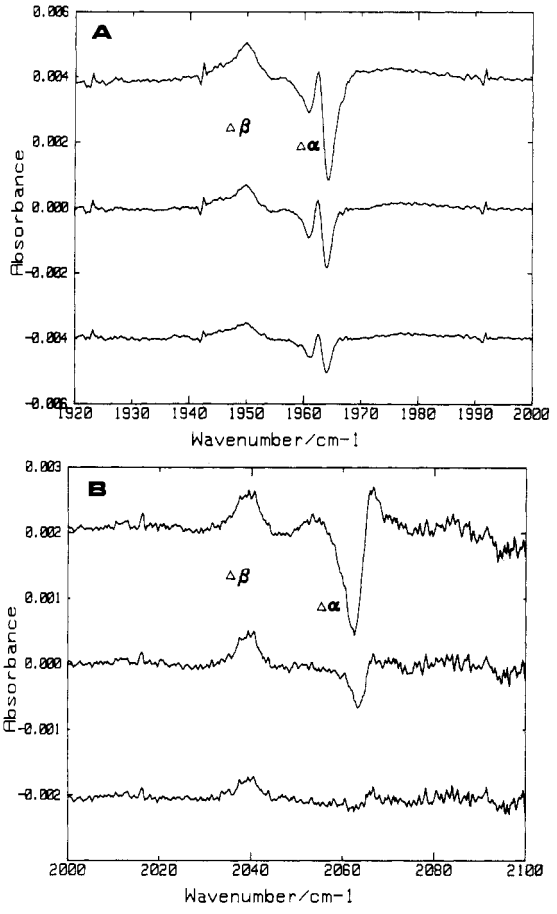

FIGURE 2: Absorbance differences of the spectra in Figure 1 for (A) FeCO and (B) CuCO. From the bottom, these are control (C, no ethanol) − (C + 0.025 M ethanol), control − (C + 0.1 M ethanol), and control − (C + 0.5 M ethanol).

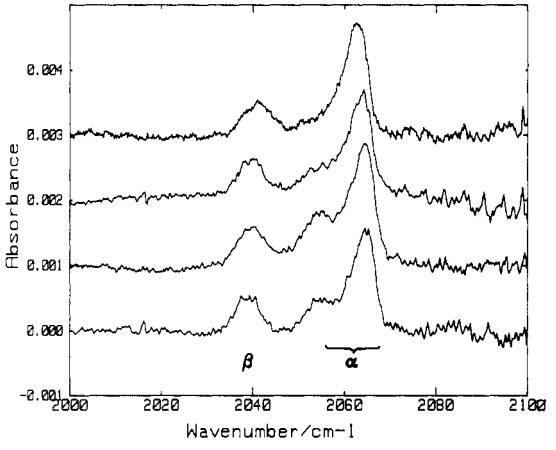

FIGURE 3: Temperature dependence of the α-CuCO band splitting in the control (no ethanol) sample. From the bottom, the temperatures are 10, 20, 30, and 60 K.

ligation at the Cu_B. These frequencies are similar to those found for the CuCO absorptions in molluscan (2063 cm^{-1}) and crustacean hemocyanins (2044 cm^{-1}) at room temperature (Fager & Alben, 1972). Therefore, while low temperature may stabilize these states, it is unlikely that freezing of the sample should induce these differences. The effects of the hydrogen-bonding solvents, water, glycerol, ethylene glycol, and ethanol, are probably due to either membrane conformational changes or local, specific hydrogen-bonding effects or a combination of both.

Molecular Model for α-Cu_BCO. The splitting of the α-CuCO absorption at low temperatures is interpreted as an exchange process in which two states are rapidly interconverted at 60 K or higher temperatures, where the rate of interconversion is fast (less than 20 fs) relative to the CO stretching frequency (2060 cm^{-1}). Note that this rate ($\gg 1.2 \times 10^{14}$ s^{-1})

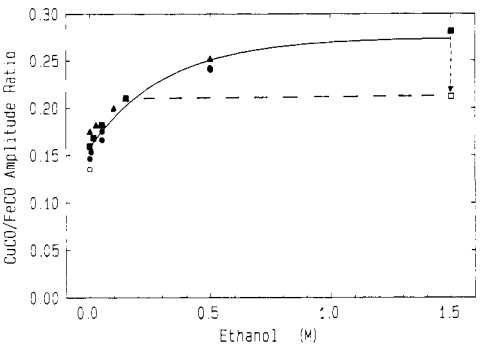

FIGURE 4: Amplitude ratio of the α-CuCO to the α-FeCO band as a function of ethanol concentration at 160 K. The dashed line at 1.5 M represents the reversibility of this reaction upon dilution of the sample (see text).

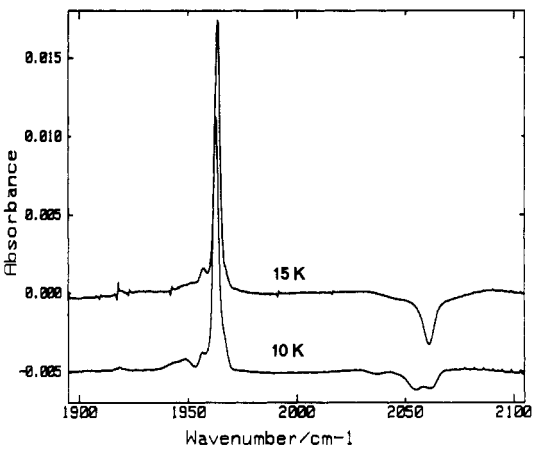

FIGURE 5: Infrared absorbance difference spectra (light minus dark) of two samples of cytochrome c oxidase in 50% (v/v) ethylene glycol at (a) 15 K and (b) 10 K. Spectrum a shows FeCO absorptions at 1952 (weak), 1957, 1964 (α), and 1968 (shoulder) cm^{-1} and CuCO absorptions at 2046 (shoulder) and 2061 (α) cm^{-1}. Spectrum b has FeCO absorptions at 1947 (shoulder), 1950, and 1962.5 (α) cm^{-1} and CuCO absorptions at 2038, 2055 (α_1), and 2062 (α_2) cm^{-1}.

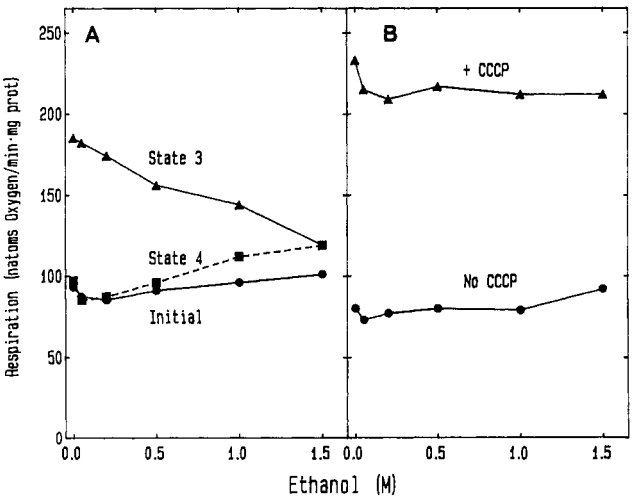

FIGURE 6: Effect of ethanol concentration on respiration of phosphorylating (A) and uncoupled (B) mitochondria oxidizing TMPD + ascorbate. Ethanol was present initially. In (A), 2–3 min after mitochondria were added (initial rate), state 3 was initiated by addition of 750 nmol of ADP. State 4 rates were measured following ADP exhaustion. In (B), phosphate was omitted from the medium and the mitochondria were uncoupled with CCCP (1.5 μM).

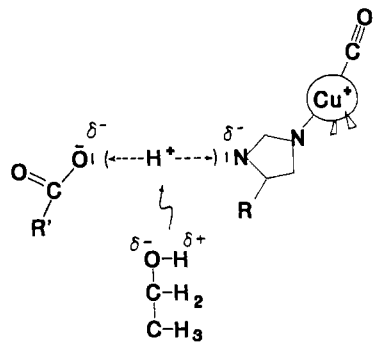

FIGURE 7: Representation of α-Cu_BCO complex ligated by at least one histidine imidazole in which the nonligated nitrogen is coupled by an H-bonded salt bridge to another ionizable group. This may be opened by an ethanol-induced change in conformation.

is much faster than diffusion-limited processes and most vibrational processes, with the exception of proton stretching vibrations. At lower temperatures, each of the states is frozen into its respective energy minimum as kT becomes small relative to the activation barrier. The transition of the α-Cu_BCO band from a doublet at low temperature to a singlet at higher temperatures implies that there is a two-state exchange process and that this exchange occurs at a rate faster than the vibrational frequency, or less than 20 fs for a 2060-cm^{-1} vibration. This indicates that the making and breaking of a hydrogen bond might be the cause of these observations. At low temperature the time spent in either state would be longer than the vibrational frequency, and two separate bands are observed. The high frequency at which this exchange process is observed (2063 cm^{-1}) rules out most types of molecular interactions, as these would oscillate at much lower frequencies. The frequency difference of 11 cm^{-1} (2054 and 2065 cm^{-1}) is too small for direct H-bonding to the CuCO but may be reasonable for H-bonding to a ligand of the Cu. A decrease of 28 cm^{-1} in the FeCO band of horseradish peroxidase was attributed to formation of a hydrogen bond between the bound CO and a water molecule (Barlow et al., 1976; Smith et al., 1983).

The near identity of the α-Cu_BCO CO vibrational frequency with that of molluscan hemocyanin CuCO and of the β-Cu_BCO frequency with that of arthropod hemocyanin CuCO suggests that coordination spheres are similar for corresponding copper centers. The copper in arthropod hemocyanin appears to be coordinated to three histidine ligands (Linzen et al., 1985; Gaykema et al., 1984). We suggest that Cu_B in cytochrome oxidase may also coordinate to one or more histidine residues.

A possible explanation of low-temperature splitting of the α-Cu_BCO is afforded by our model described in Figure 7, in which we suggest that an ionizable group such as carboxyl from aspartate, glutamate, or a_3-heme propionate may be H-bonded to a histidine imidazole ligand to Cu_B. The proposed remote H-bond could then vibronically affect the CuCO stretching frequency in a delicate fashion, depending upon whether the proton was more closely associated with the imidazole nitrogen or with the carboxyl group to which it is H-bonded in this model. At high temperatures, this would be a "normal" H-bond, with rapid exchange between two states, as is possible for a proton in an H-bonding field. At low temperatures, either of the two states may be stabilized in which the proton is associated either with the imidazole nitrogen or with its associated carboxyl group. The appearance of two stable states at low temperatures may limit the H-bond donor to an ionizable species such as a carboxyl group. It appears unlikely that a nonionizable H-bond donor could lead to more than a single ground state at lower temperatures. Rotation of an alcoholic OH around a C–O bond could produce two states (alcoholic H-bond acceptor or donor), which could only exchange slowly relative to the carbonyl stretching frequency (rotational motions are slow relative to CO vibrational stretching motions) and thus could not produce an unsplit carbonyl absorption band as we observe at higher temperatures (80 K). The low-temperature splitting of a CuCO is not observed in the β-form of cytochrome oxidase, which must therefore lack this type of H-bonded interaction. It is entertaining to suggest that such a mobile proton (Figure 7) in the α-form of cytochrome oxidase may be involved in respiratory control and in proton pumping. This might provide a molecular mechanism for the effects of pH and transmembrane potential on respiratory control of electron transfer at the cytochrome *a*–cytochrome a_3 level (Gregory & Ferguson-Miller, 1989). The presence of an exchangeable proton within the a_3Fe–Cu_B pocket is required for the complete reduction of dioxygen to water. Our spectral evidence for an exchangeable hydrogen in the vicinity of the Cu_B could be important for understanding the mechanism of proton transfer to dioxygen during the catalytic cycle. This exchangeable proton, being on a Cu_B ligand, could be available for dioxygen reduction.

Effects of Ethanol and Other Solvents. (*A*) *α-Forms.* We further suggest a specific mechanism of ethanol effects on α-cytochrome *c* oxidase. Replacement of the putative H-bonding carboxyl group by ethanol may explain the loss of low-temperature splitting and the band broadening we observe at the α-Cu_BCO absorption. The similarity of the α-Cu_BCO absorption band at low temperature in the presence of ethanol with higher temperature data from control samples suggests that the ethanol disorders the α-structure in a manner similar to thermal effects. This is observed as an increase in bandwidth of the unsplit α-FeCO band and can be thought of as due to an increase in local "fluidity" (or disorder) at the a_3Fe–Cu_B site. Several laboratories have reported that addition of ethanol to mitochondrial membranes increases their fluidity at higher temperatures (Trudell et al., 1973; Rubin & Rottenberg, 1982). Vanderkooi and associates have investigated the effects of several amine and 1-alkanol local anesthetics (which have also been reported to increase membrane fluidity) on mitochondrial electron transport (Chazotte & Vanderkooi, 1981). They have shown that several enzymes, including cytochrome *c* oxidase, are inhibited by these local anesthetics. In analyzing thermodynamic parameters that characterize the interaction of anesthetics with cytochrome oxidase, they suggested that inhibition of enzyme activity was caused by a reversible perturbation of protein comformation, to a degree much smaller than that required for thermal denaturation (Vanderkooi & Chazotte, 1982). This suggests that an ethanol-induced increase in protein fluidity, in addition to specific binding near the metal sites in cytochrome oxidase, may cause changes that we observe in the α-form of the enzyme.

(*B*) *β-Forms.* It is not certain from our present studies whether the β-forms of cytochrome oxidase are converted to the α-form or if they are altered in such a way that they no longer bind CO. The latter could be due to a modification of either the a_3Fe or Cu_B site so as to prohibit the binding of CO (and O_2). In mitochondria isolated from four separate beef hearts, the β-forms of the control samples (no ethanol) represent only 15–22% of the total enzyme, making it difficult to quantitate the precise degree of change. However, our results do indicate that the effects are strictly controlled by the concentration of ethanol.

The loss of β-forms observed by infrared spectroscopy with increasing ethanol concentration could be due to several possible causes. One, the β-forms could be converted into the α-form. Two, the β-forms could lose their ability to bind CO (and by inference O_2) in the presence of ethanol. Ethanol bound to a_3Fe(III), as in hemoglobin and myoglobin (Brill et al., 1976), would stabilize the oxidized form and thus prevent binding of carbon monoxide. Thayer and Rubin (1986) reported that liver mitochondria from ethanol-fed rats showed a 50% decrease in cytochrome oxidase activity that was apparently due to half the protein being present in a nonfunctional state, not containing hemes *a* and a_3. It is a possibility that the β-forms of cytochrome oxidase become inactivated by an ethanol-mediated disruption of the a_3-heme and hence lose the ability to bind CO. If this were the case, it would strongly imply that ethanol was directly interacting with the protein, in addition to any effect on the surrounding membrane. The β-forms probably do not lose their hemes, as an effect this drastic would most likely not be selective for only one form of the protein. Since the α-form of cytochrome oxidase retains its ability to reversibly bind CO to both the a_3Fe and Cu_B atoms, ethanol cannot be binding directly to the metals. We do not yet know if the ethanol-induced alterations of the β-forms of cytochrome oxidase are reversible and so cannot say the same for the β-a_3Fe and β-Cu_B sites. A third possibility for lack of observation of β-forms of oxidase–CO in the infrared spectra is that the β-FeCO signal may relax extremely fast following photodissociation so that the β-forms are not observed in the light/dark difference spectra even at 10 K. This would suggest that the photodissociated CO may no longer be stabilized by binding to the β-Cu_B site. Any of the reasons suggested for possibilities two and three imply that the β-forms of the enzyme have become inactivated by the alcohol.

Effects of Ethylene Glycol and Glycerol. The local environments of Cu_BCO in the presence of 50% ethylene glycol were much more variable (Figure 5). It would appear that in the second sample cited above the enzyme froze with a broad distribution of Cu_BCO structures, whereas the first sample froze into discrete Cu_BCO states stabilized by the low temperature, with additional structural states becoming available as the temperature increased. This may be caused by variable penetration of ethylene glycol within the membrane and/or by how slowly the sample is frozen. Ethylene glycol at 50% or higher concentration has been used extensively to produce a "frozen glass" for low-temperature studies of biological systems. Our observations suggest that membrane-bound enzymes may be modified by ethylene glycol and that experimental results may need to be interpreted with caution. Conversely, glycerol-extracted mitochondria exhibit low-temperature photodissociation difference spectra similar to those observed with aqueous mitochondrial pellets. It appears that glycerol is not incorporated into cytochrome oxidase under these conditions.

Physiological Effects of Ethanol. Ethanol is a weak anesthetic (Seeman, 1972) that at high concentrations causes structural changes in biological membranes, including membrane expansion and increased fluidity [Trudell et al., 1973; see Rubin (1987) for a recent review]. In isolated mitochondria ethanol has been reported to act as a weak uncoupler, stimulating ATPase activity and respiration under nonphosphorylating (state 4) conditions but also inhibiting respiration under phosphorylating (state 3) conditions (Rottenberg et al., 1980). This uncoupling effect becomes more pronounced at higher temperatures, with no respiratory control evident at 45 °C in the presence of 1 M ethanol (Rottenberg et al., 1980).

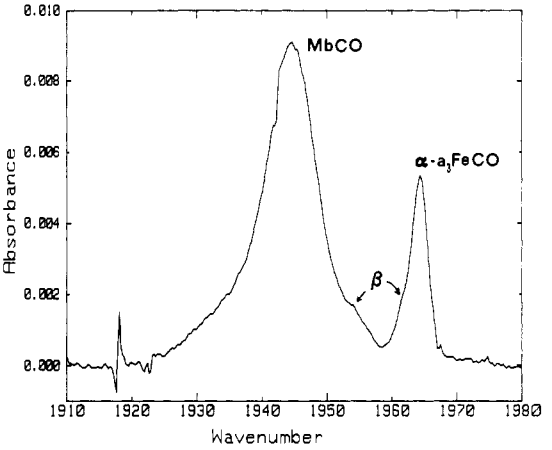

FIGURE 8: Infrared (FeCO) absorbance spectrum of CO-saturated aqueous pellet of rat heart myocytes at 15 °C. An aerated sample was used as the reference and the spectrum was corrected for water, water vapor, and base line. Myoglobin is observed at 1944.6 cm^{-1}, the β-forms of cytochrome *c* oxidase are shoulders at 1954.3 and 1961.5 cm^{-1}, and the α-form of cytochrome oxidase is at 1964.4 cm^{-1}.

Uncoupler-stimulated respiration is the most direct measurement of cytochrome oxidase activity, since it is only dependent on uncoupler effectiveness in collapsing the proton electrochemical gradient across the inner membrane. Besides oxidase activity, ADP-stimulated respiration depends on three other transmembrane proteins: the phosphate and adenine nucleotide transporters and the ATP synthetase. The mitochondrial structural changes induced by ethanol (Waring et al., 1982) may affect these membrane proteins in addition to cytochrome oxidase and probably account for the reduced state 3 oxygen uptake. The observation that uncoupled respiration is 27% greater than state 3 respiration (Figure 6) suggests state 3 is limited by one of these proteins involved in ATP synthesis rather than by the oxidase itself. The effect of ethanol on cytochrome oxidase at 24 °C appears to be limited to a 10% reduction in activity at 50 mM ethanol (Figure 6). This may correspond to loss of β-cytochrome oxidase or to modification of the Cu_B site of the α-form to convert it to a less active structure. Further increase in ethanol concentration produced no further loss of uncoupled cytochrome oxidase activity. Nonstimulated oxygen uptake rates appear to be controlled by the mitochondrial proton electrochemical potential (Gregory & Ferguson-Miller, 1989), and inhibition of nonstimulated respiration (Figure 6) could result from ethanol effects on available proton influx pathways (leaks, ATP synthetase, phosphate carrier) rather than on cytochrome oxidase itself. In contrast to our results, Cederbaum et al. (1974) reported low ethanol concentrations (6–80 mM) to have no effect on ascorbate oxidation or ascorbate-linked respiratory control.

Robinson et al. (1985) have reported that when purified lipid-depleted enzyme was dissolved in different detergents, the rates of electron transfer depended upon the structure of the polar head groups and the length of the hydrocarbon tail. They interpreted this to suggest that there are two forms of cytochrome oxidase, one significantly more active than the other. These may be related to the α- and β-structural forms that we observe.

We have observed the β-forms at greater concentration in heart tissue and myocytes. With these samples we see the β-FeCO absorptions as shoulders on the more intense myoglobin FeCO and cytochrome oxidase α-FeCO bands. In Figure 8 we show a CO-saturated rat heart myocyte spectrum

at 15 °C, where an aerated myocyte sample is used as the reference. The observation of the β-forms of cytochrome *c* oxidase in myocytes at room temperature indicates that they are present under physiological conditions and that they are not artifacts of freezing. Ethanol changes the structure of the O_2-binding site of cytochrome *c* oxidase for both the α- and β-structures. The relative distribution of these forms may be important for respiratory control but does not appear to affect the maximum rate of O_2 consumption. One of the major questions left by these studies of the α- and β-forms of the carbon monoxide complexes of cytochrome *c* oxidase is whether these forms are interconvertible and represent an aspect of the metabolic control of cytochrome oxidase activity or whether they are genetically regulated isozymes with different amino acid sequences and possibly different coordination spheres around the iron and copper oxygen-binding sites (Fiamingo et al., 1988). The fractional amounts of β-forms of cytochrome *c* oxidase are considerably greater in heart tissue and myocytes than in isolated mitochondria. Studies of these tissues will help to answer the question of whether the β-forms become incapable of binding carbon monoxide in the presence of ethanol or, alternatively, whether the β-forms may be quantitatively converted into the α-form of cytochrome *c* oxidase.

The effects that we observed of ethanol on cytochrome oxidase structure appear to be relevant for physiological function in vivo and also should provide a caution for in vitro study of mitochondrial energetics. We observed 40% disappearance of the β-forms at 0.05 M (0.23%) ethanol. Most states consider 0.022 M (0.1%) blood alcohol levels to indicate legal intoxication, and levels as high as 0.1 M have been seen in some alcoholic patients. These results suggest a direct molecular basis for some of the dysfunctions associated with ethanol toxicity and provide a pathway by which some of these problems may be better understood. Our results further suggest caution when using ethanol as a carrier for water-insoluble agents in view of its significant effects on respiratory chain activity.

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