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Formate bound to cytochrome oxidase can be removed by cyanide and by reduction

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Abstract

Using ^{14}C-radiolabeled formate we have found that the rapid form of oxidized cytochrome oxidase can bind up to 1 mol of formate. Treatment of this formate-ligated enzyme with excess cyanide releases 97% of the radiolabel while reduction of formate-labeled enzyme with NADH + ruthenium releases 80–85% of the radioactivity. These data are most simply interpreted by assuming that formate binds to the heme iron of cytochrome a_3.

Keywords: Cytochrome oxidase; Formate binding; Fast conformer; Slow conformer

1. Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1; CcO) from beef heart is the multi-subunit terminal enzyme of the mitochondrial respiratory chain. The enzyme contains 4 redox-active metal sites including 2 heme *a* (cytochromes *a* and a_3) and 2 copper sites (Cu_A containing 2 copper atoms, and Cu_B) [1,2]. Four electrons from ferrocytochrome *c*, the physiological reductant, are transferred to cytochrome *a* via Cu_A. Subsequently cytochrome *a* delivers the electrons to the binuclear center comprised of cytochrome a_3 and Cu_B [3]. This latter center is the site where dioxygen is reduced to water and also appears to be the site responsible for proton translocation [4]. The binuclear center is also the site with a high affinity for exogenous ligands site though both cytochrome *a* and Cu_A also react with such added reagents when at high concentrations [5].

The ability of formate to inhibit cytochrome oxidase was first reported by Nicholls [6] who studied the effect of this reagent in raising the steady-state level of reduction of cytochrome *c* in sub-mitochondrial particles. Inhibition was attained slowly and this allowed Nicholls to demonstrate that formate did not react with the reduced enzyme. Further a comparison of the inhibition produced by azide first in the presence and then in the absence of formate led Nicholls to propose that these two inhibitors were reacting at the same site.

Subsequently Nicholls [7] reported that formate produced a blue shift of the Soret maximum of submitochondrial particles and that this shift was

Abbreviations: Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; DM, dodecyl-β-D-maltoside; Tris, Tris-[hydroxymethyl]aminomethane.

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only found in oxidized and partially reduced but not in fully reduced particles. These data are consistent with formate binding to ferric heme iron for ferrous hemes do not readily bind anionic ligands. Furthermore, Nicholls [7] noted that cyanide could compete effectively with formate for the ligand binding site.

The native, oxidized form of cytochrome oxidase exists in at least two conformers, called 'fast' and 'slow' [8]. They are distinguished by differences in cyanide reactivity, the wavelength of the maximum Soret absorbance and the presence in the slow form of epr resonances which are lacking in the fast form [8,9]. The fast form can convert spontaneously to the slow form typically in response to mildly acid conditions [8]. We have reported that this fast to slow conversion can also be accomplished by addition of formate [10] and that formate was the only compound amongst a large number tested that was effective. We also showed that fast cytochrome oxidase could bind close to stoichiometric quantities of formate.

In pursuing the nature of the interaction of formate with CcO, we felt it was important to establish if the binding of formate to cytochrome oxidase was eliminated by the chemical treatments reported by Nicholls to interfere with formate binding, namely addition of cyanide and reduction of the heme centers.

We have confirmed our previous observation that cytochrome oxidase binds essentially stoichiometric quantities of formate and find that both reaction with cyanide and reduction of the enzyme reduces the amount of bound formate substantially, to almost zero in some cases.

2. Materials and methods

2.1. Materials

Solubilized beef heart cytochrome *c* oxidase was prepared using the modified Hartzell-Beinert method [8]. The final precipitate was dissolved in 50 mM Hepes (pH 8.0), containing 0.1% w/v, DM for storage. Before use, stock enzyme was diluted into 50 mM Tris-sulfate buffer (pH 8.0), containing 0.1% DM followed by concentration using Amicon Centriflo ultrafiltration cones; this procedure was repeated an additional 2 times. This enzyme had an absorbance maximum in the Soret band at 422 nm.

2.2. Methods

Conversion of enzyme from the fast to slow form [10] was achieved by incubation with excess formate. For example stock enzyme was diluted to give 1.0 ml of a 50-μM solution in 50 mM Tris-sulfate, 0.1% DM and the UV-vis spectrum recorded. Then 10 μl. of a freshly-made, 1 M formate solution (in 200 mM Tris-sulfate, pH 8.0, 0.4% DM buffer) was added to give a final formate concentration of $\sim$ 10 mM. To each ml of enzyme solution 10 μCi, sodium [^{14}C]formate (Amersham Corp.) was added. After 2 days incubation at 0°C, the enzyme exhibited the Soret absorption spectrum and cyanide reactivity of the slow form [8,11].

2.3. Preparation of cyanide enzyme

Formate-treated enzyme was divided into two aliquots. One was put aside as the control and the other was treated with various concentrations of cyanide (8–40 mM; stock solution 0.8 M, pH 9.0) for 2 days on ice.

2.4. Preparation of reduced enzyme

Formate-treated enzyme was divided into two aliquots as before and one aliquot was reduced anaerobically using 0.73 mM NADH with 87 μM hexaammineruthenium (III) chloride (Alfa) as mediator. In these experiments enzyme was made anaerobic in a Thunberg-type using several cycles of argon and vacuum. The ruthenium solution was added to the enzyme and the NADH placed in the sidearm; the process of anaerobiosis was repeated, the enzyme placed under argon and then mixed with the NADH. The reaction mixture was maintained at 0°C in the dark for 2 h. Optical measurements showed that the enzyme had become fully reduced after 10 min. The concentrations of the stock solution of NADH (in 50 mM Tris-sulfate, pH 7.6) were determined using a molar absorbance of 6220 M^{-1} cm^{-1} at 340 nm. The stock solution of ruthenium (ca. 12.5 mM) was prepared in water and a suitable volume added to the enzyme to obtain the desired final concentration.

2.5. Column chromatography

The following conditions were found to minimize turbidity of the enzyme after elution from the col-

umn. A Bio-Gel P6 column (Bio-Rad, 1.5 × 12 cm, 20 ml bed volume) was used to separate bound and free formate. The column was equilibrated and eluted with 50 mM Tris-sulfate, pH 8.0–0.3% DM buffer at 4°C. In the experiments with reduced enzyme, the buffers applied on the column were purged with nitrogen gas (ultra pure, Trigas) during both the equilibration and elution period. The flow rate was adjusted to about 1 ml per min. Before applying to the column the DM concentration in the sample was adjusted to 0.6% and a 0.5-ml portion was loaded onto the column; 0.8-ml fractions of column elute were collected. The absorbance of each fraction was monitored at its appropriate Soret maximum. Then the aliquots were immediately dispersed in scintillation fluid (Sigma-Fluor) and the resulting samples counted using a Beckman LS 3801 liquid scintillation system.

Cyanide kinetics were determined at pH 7.4 in 0.1 M Hepes buffer containing 0.1% DM by following the increase in absorbance at 428 nm in the presence of 8 mM cyanide. Catalytic activity was measured by following the oxidation of reduced cytochrome *c* at 550 nm in 50 mM phosphate buffer (pH 6.65) containing 0.5% Tween 20. The spectral, radio-isotope and kinetic data were analyzed using Igor Pro (Wave Metrics, Lake Oswego, OR).

3. Results

3.1. Preparation of formate-treated enzyme

Incubation of 50 μM CcO with 10 mM formate for 2 h led to a shift of the Soret band from 422 nm to 418 nm. Incubation was continued for 2 days but the position of the Soret band did not shift further. At this time the 655 nm band was slightly red-shifted and the alpha band slightly decreased while the beta and 830 nm bands were not significantly changed. Following dilution by 10× for spectrophotometric analysis, almost 98% of the enzyme reacted slowly with 8 mM cyanide ($k_{obs} = 1 \times 10^{-4}$ s^{-1}).

The catalytic activity of both the formate-treated enzyme and the control decreased during the incubation. The control enzyme lost 0–20% activity in different experiments. The formate-treated enzyme lost 60–70% of the zero time activity within 2 h; the activity then remained constant over the balance of the two-day incubation period.

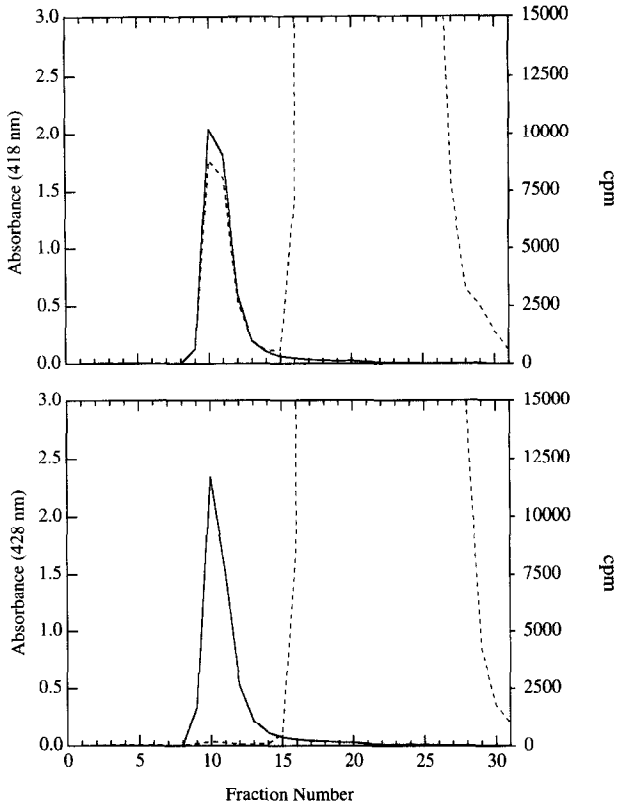

Fig. 1. Comparison of the elution profiles for formate-treated cytochrome oxidase (upper panel) and formate-treated enzyme subsequently reacted with potassium cyanide (lower panel). Unbroken line, absorbance changes at 418 nm (upper panel) or 428 nm (lower panel); dotted line, radioactivity data. Note that the axes have been scaled so that the peaks in the absorbance and radio-isotope data overlap. Additional details are given in Section 2.

3.2. Cyanide can displace formate

To evaluate the ability of cyanide to displace formate, formate-treated enzyme prepared as above was reacted with 40 mM cyanide for an additional 2 days. The Soret maximum had shifted to 428 nm at the end of this period.

Fig. 1 shows the elution profiles for control and cyanide-enzyme from this experiment. In the case of the control 0.92 mol of formate were found to elute with the enzyme. The range of values from a number of experiments was 0.83–0.96 mol formate bound per mol of enzyme. The clear separation of the bound

and free formate and the close match of the radioactivity and enzyme profiles implies that bound formate dissociates slowly on the time scale of the chromatography. After reaction with cyanide the amount of formate bound was reduced to 0.02 mol. In experiments with lower concentrations of cyanide (8 mM), it was found that cyanide does not completely displace all of the formate; the estimate of residual bound formate was approx. 0.3 mol.

3.3. Reduction of formate-treated enzyme leads to loss of formate

Formate-treated cytochrome oxidase was reduced anaerobically using excess NADH in the presence of Ru(III) as a mediator. Reduction was rapid and optical spectra recorded after 25 min and 2 h were the same and identical to that of enzyme reduced in the absence of formate.

Elution profiles for both heme absorbance and radiolabeled formate are shown in Fig. 2. The upper panel shows the profile for the control which had not been reduced. Again there is very good overlap of the absorbance and radioactivity; the number of moles of formate per oxidase calculated from this data was found to 0.96.

The lower panel shows the elution profile obtained with reduced enzyme. Clearly a considerable amount of formate has dissociated from the enzyme; the residual bound formate is 0.19 mol per mol of enzyme in this experiment (range = 0.15–0.2).

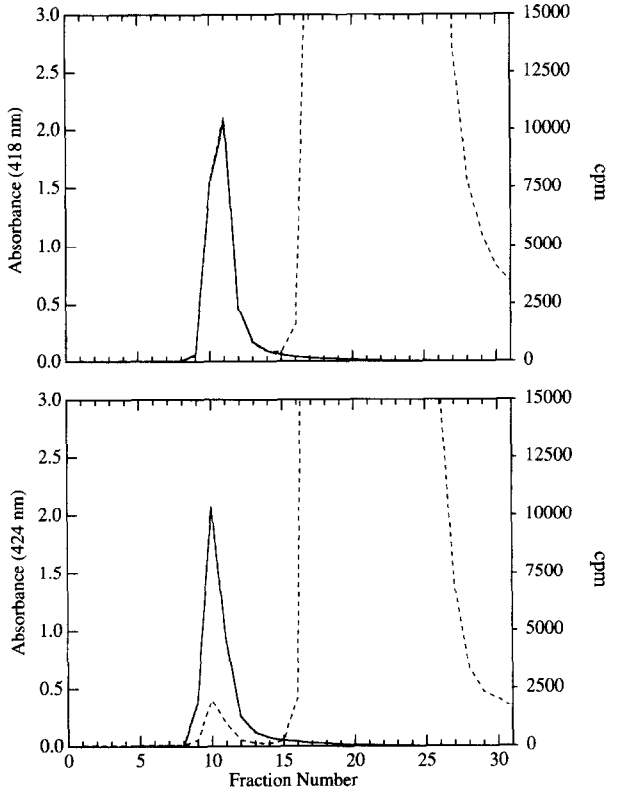

Fig. 2. Comparison of the elution profiles for formate-treated cytochrome oxidase (upper panel) and formate-treated enzyme after reduction (lower panel). unbroken line, absorbance changes at 418 nm (upper panel) or 424 nm (lower panel); dotted line, radioactivity data. The enzyme fractions were allowed to stand in air for 2 h before recording spectra to ensure that they were fully converted to the oxidized state. Note that in the leading peak of the upper panel both sets of data overlap almost exactly. Other details are given in the legend to Fig. 1.

4. Discussion

The data reported here, taken with those published previously, are most simply explained if formate is a ligand to the heme iron of cytochrome a_3. The wavelength maximum of the Soret is modified, the mcd is unchanged [10], the stoichiometry of formate binding approaches 1 and the formate can be displaced both by cyanide and by reduction. Each of these observations is consistent with such an interpretation. However, it should be noted that none of these observations constitutes proof that formate is coordinated to the heme; similar phenomena might be observed if formate is bound to the binuclear center in such a way as to significantly perturb the structure of cytochrome a_3 and for the binding to be responsive to ligation of the heme by cyanide.

While a blue shift of the Soret is normally associated with a low-spin to high-spin transition of the heme iron, there is no evidence that cytochrome a_3 is low-spin in the absence of added ligands. Thus this blue-shifted Soret is most likely due to a weakening in the ligand-field strength of a ligand that is already weak-field. Cytochrome a_3 is unequivocally the target as mcd spectra clearly show that the optical spectrum of cytochrome a is unaffected by such treatment [10]. Likewise the elimination of formate binding following addition of cyanide is a clear indication that formate interacts with a_3 because cyanide is generally accepted as an a_3 ligand. The need to

displace the formate in this reaction is then a simple explanation for the reduced rate of reaction of cyanide with enzyme that has been reacted with formate. The loss of formate following reduction is also consistent with binding to heme. While ferric heme has a formal charge of +1 and is thus frequently found associated with an anion, reduced heme binds anionic ligands weakly, if at all. Cyanide is a clear exception to this generalization but even with cyanide the affinity is 100–1000 × weaker than with ferric heme; furthermore cyanide can draw on π orbitals to help in binding to ferrous iron.

At the same time the appearance of the epr signals at $g = 12$ [10] and at 2.95 [9] after addition of formate imply that the magnetic coupling [12–14] between a_3 and Cu_B is retained. These epr signals arise from an integer spin paramagnet which requires that either a_3 be in the ferrous state (which is clearly not the case) or that the integer spin is the product of coupling between the $S = 5/2$ high spin heme of a_3 with the $S = 1/2$ Cu_B center. This coupling is most readily understood if formate functions as a bridge between these two metal centers. The X-ray structures certainly suggest that there is an available coordination site present on the proximal side of Cu_B which appears to be coordinated by three histidines, each of which is distal to the iron [2]. Thus from a stereochemical point of view a bridging ligand would seem feasible. Note, however, that the epr data alone cannot tell whether the strength of the coupling has changed following formate addition, though published data suggest that strong coupling persists [13].

The intrinsically slow form of CcO shares many physical properties and the reduced chemical reactivity exhibited by the formate-treated enzyme. This raises the important question as to the identity of the endogenous ligand responsible for the spontaneous conversion of fast enzyme to slow enzyme. An obvious candidate would be the carboxylate side-chain of an aspartate or glutamate residue within the vicinity of the binuclear center. This possibility appears to have been unequivocally ruled out for the case of Glu286, for mutation to asparagine in the cytochrome *bo* of an *Escherichia coli* strain [15] and to glutamine in the aa_3 of *Rhodobacter sphaeroides* [16] does not eliminate the fast to slow conversion that occurs in these members of the oxidase super-family. Other carboxylate groups are present in the extra-membrane loop regions [1] but, in the absence of large structural changes, these residues are just too far away to be serious candidates.

The reaction of formate with cytochrome oxidase is complex with several kinetic phases and Baker and Gullo [17] suggest an upper value of about 0.1 M^{-1} s^{-1} for the association rate constant. When this value is combined with an apparent dissociation constant of 0.3–0.5 mM [17] (W. Li and G. Palmer, unpublished data), we obtain a value of ca. 5×10^{-5} s^{-1} for the dissociation constant. This value is sufficiently small that little or no formate should dissociate from the enzyme during the column chromatography associated with our experiments. This expectation is consistent with our observations (Figs. 1 and 2) that the radioactivity profile of the bound formate accurately overlaps that of the absorbance profile of the enzyme.

The amount of bound formate never exceeded 0.96 mol per mol of enzyme. A value of ca. 0.5 mM for the dissociation constant predicts that the enzyme will be 95% saturated under our experimental conditions. Thus the maximum yield that we have observed is 1.01 mol formate bound, a value impressively consistent with the notion that there be only a single site for the binding of this ligand. Whereas the displacement of formate by 40 mM cyanide appears to be essentially complete, some residual formate was consistently observed in the enzyme which had been reduced. Whether this is due to a low affinity of reduced enzyme for formate or represents the technical difficulty in ensuring complete anaerobiosis during the chromatographic separation remains to be established.

There is an apparent anomaly in data obtained using 8 mM cyanide to displace formate. Under these conditions essentially all of the enzyme reacts with cyanide, as gauged by the kinetics of ligation, but only 70% of the formate is liberated from the enzyme. However the two sets of data were obtained under somewhat different conditions with the kinetic experiment being conducted with a reaction mixture one-tenth the concentration of the chromatographic experiment. In the former case the formate concentration is comparable to the K_d for formate binding and thus the reaction with cyanide is promoted. Raising the cyanide concentration to 40 mM is sufficient to ensure complete release of formate under the incubation conditions employed.

While the formate-treated enzyme exhibited a smaller catalytic activity than control enzyme, this difference developed during the first 2 h of incubation during which time most of the spectral changes occurred; no further activity was lost over the balance of the two-day incubation. This behavior suggests that the difference in activity is a characteristic property of the formate enzyme and does not reflect any non-specific denaturation.

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