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Formamide probes a role for water in the catalytic cycle of cytochrome *c* oxidase☆

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Abstract

Formamide is a slow-onset inhibitor of mitochondrial cytochrome *c* oxidase that is proposed to act by blocking water movement through the protein. In the presence of formamide the redox level of mitochondrial cytochrome *c* oxidase evolves over the steady state as the apparent electron transfer rate from cytochrome *a* to cytochrome a_3 slows. At maximal inhibition cytochrome *a* and cytochrome *c* are fully reduced, whereas cytochrome a_3 and Cu_B remain fully oxidized consistent with the idea that formamide interferes with electron transfer between cytochrome *a* and the oxygen reaction site. However, transient kinetic studies show that intrinsic rates of electron transfer are unchanged in the formamide-inhibited enzyme. Formamide inhibition is demonstrated for another member of the heme-oxidase family, cytochrome *c* oxidase from *Bacillus subtilis*, but the onset of inhibition is much quicker than for mitochondrial oxidase. If formamide inhibition arises from a steric blockade of water exchange during catalysis then water exchange in the smaller bacterial oxidase is more open. Subunit III removal from the mitochondrial oxidase hastens the onset of formamide inhibition suggesting a role for subunit III in controlling water exchange during the cytochrome *c* oxidase reaction.

Keywords: Cytochrome *c* oxidase; Formamide; Water; Catalytic cycle; Subunit III; Electron transfer; Water cycle; Proton gathering

1. Introduction

Cytochrome *c* oxidase belongs to the heme-copper oxidase super-family and has four redox centers: Cu_A, cytochrome *a*, cytochrome a_3 and Cu_B. Cytochrome *c* oxidase catalyzes electron transfer from cytochrome *c* to O_2 [1,2]. During the catalytic cycle, four electrons are transferred to dioxygen as eight protons are taken up from the mitochondrial matrix space. Electrons are delivered from cytochrome *c* one at a time to the Cu_A center within subunit II that serves as the initial electron-accepting site of the oxidase. Electrons are then passed via cytochrome *a* to the binuclear, cytochrome a_3-Cu_B site that functions as the oxygen reaction site. Four protons are involved in the reduction of dioxygen to form water, and another four protons are pumped out from the mitochondrial matrix space to the inter-membrane space to produce a proton motive force, which ultimately contributes to ATP production catalyzed by the H^+-linked ATP synthase.

Just as there are particular electron transfer pathways for delivering reducing equivalents from cytochrome *c* to oxygen that have been defined within cytochrome *c* oxidase by a variety of transient spectroscopy experiments (e.g., [3–5]) efforts have been made to identify particular proton translocation pathways (e.g., [6]) that can deliver protons for chemical reduction to make water and that can be pumped across the membrane for energy conservation [7,8]. The high resolution structures of a number of heme-copper oxidases reveal distinct physical channels that are proposed as possible proton conduits (e.g., [9]). Further definition has been given to two proton channels by mutagenesis experiments. The D-channel begins with a conserved aspartic acid residue that is found at the cytosolic-, or matrix-exposed surface of subunit I [10]. The proposed proton pathway then proceeds via a series of water molecules that lead to a glutamic acid residue located between the heme groups and near the centre of the lipid bilayer. Proton transfer from this glutamic acid to the oxygen reaction site involves a distance of about 10 Å′ to the oxygen reaction site, and further to exit the

☆ TMPD: *N, N, N′, N′*-tetramethyl-*p*-phenylendiamine; Formamide-inhibited cytochrome *c* oxidase: because formamide inhibition of cytochrome *c* oxidase requires catalytic turnover, the formamide-inhibited enzyme refers to the enzyme obtained after catalytic turnover in the presence of formamide.

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protein at the outer surface. There are no appropriate amino acid side chains in this space, nor are waters found in the known crystal structures. However, molecular dynamics studies have led to the view that a chain of water molecules could occupy the space above the glutamic acid residue and lead protons to the magnesium ion located at the outer surface-exposed interface of subunits I and II, or to the propionic side chain of the heme of cytochrome a_3. The K-channel is less well defined but includes a conserved lysine residue and a few water molecules to transfer protons to the binuclear center. The D-pathway is proposed to deliver protons that are pumped across the membrane and some of the protons consumed during O_2 reduction, whereas the K-pathway is involved only with the substrate protons used for O_2 reduction [11].

Water molecules in cytochrome *c* oxidase have attracted considerable interest because water is the product of oxygen reduction [12] and is involved in the hydrogen bond networks that are proposed to play an important role in proton transfer [9,13]. Computation and simulation based on available crystal structures of cytochrome *c* oxidase suggest that bound water molecules inside the enzyme form several water chains, which contribute to proton translocation through cytochrome *c* oxidase [14–16]. These water chains are observed in the D- and K- pathways in the structures of the oxidase and are composed of relatively long-lived water molecules in these positions. Water chains are also found in computational studies to occupy spaces above the key glutamic residue as mentioned above. These water molecules are not observed in the crystallographic studies because of their dynamic nature, but their position may persist in a manner consistent with a role in catalysis. The existence of functionally important water chains within cytochrome *c* oxidase are consistent with previous experiments, which have shown that both high osmotic and hydrostatic pressure are able to block electron transfer from cytochrome *a* to the binuclear center of cytochrome *c* oxidase, thus indicating the importance of the enzyme's hydration state [17,18].

Studies on formamide as an inhibitor of cytochrome *c* oxidase have led to the proposal of a water channel within cytochrome *c* oxidase that is coupled to the electron transfer and proton translocation reactions catalyzed by cytochrome *c* oxidase [19]. Formamide is a small chemical with a molecular volume that is twice that of water [20], and may prove a useful tool in augmenting the experimental evidence of water's involvement in the catalytic process of an enzyme such as cytochrome *c* oxidase. Formamide has been proposed to block the water channel and thereby inhibit the catalytic activity of cytochrome *c* oxidase [19]. In the present study, we have investigated the effect of formamide on the redox state of cytochrome *c* oxidase during steady-state turnover. Our results are consistent with the suggestion that formamide inhibition results from blocking electron transfer from cytochrome *a* to the cytochrome a_3-Cu_B center. However, single turnover studies of formamide-inhibited, reduced cytochrome *c* oxidase reacting with O_2 do not show a direct effect of formamide on intrinsic rates of electron transfer. Formamide is also effective as a turnover-dependent inhibitor of the cytochrome caa_3 complex from the bacterium *Bacillus subtilis*. Preparations of *B. subtilis* cytochrome caa_3 are composed of subunits I and II, which are the two major conserved subunits amongst the heme-copper oxidase family. Formamide-inhibition of the steady-state turnover of *B. subtilis* cytochrome *c* oxidase reaches a similar level as that observed with the mitochondrial oxidase over a similar range of formamide concentration. However, inhibition of *B. subtilis* cytochrome *c* oxidase activity is established much quicker than with the mitochondrial enzyme. In addition, onset of formamide inhibition occurs much more quickly with the subunit III-depleted version of the mitochondrial oxidase. If formamide inhibits by blocking a water channel then access to this channel is more open with the bacterial oxidase than in the mitochondrial enzyme and subunit III may have a role in controlling the access of water to the oxidase interior.

2. Material and methods

Horse heart cytochrome *c* was from Sigma-Aldrich Chemicals (St. Louis, MO) and used without further purification. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), or BioShop Canada Inc. (Burlington, ON), and were of the usual analytical grade. Lauryl maltoside (n-dodecyl-β-D-maltoside) was obtained from Anatrace, Inc. (Maumee, OH). Redistilled formamide was the kind gift of Dr. J. A. Kornblatt (Concordia University, Montreal, PQ).

Beef heart cytochrome *c* oxidase was prepared according to the method of Kuboyama et al. [21]. This procedure results in a preparation in which the oxidase is largely in the resting state. We opted to use this approach because it allows us to observe the resting to pulsed transition as turnover commences. Stock solutions containing 0.38 mM cytochrome aa_3 were stored in liquid nitrogen in 0.1 M sodium phosphate buffer, pH 7.0, with 0.5 mg/mL lauryl maltoside. Cytochrome caa_3 from *Bacillus subtilis* was purified according to the method of Henning et al. [22].

Subunit III-depleted bovine cytochrome *c* oxidase was prepared by a variation of a method described previously [23]. The oxidase (1.3 mg) was coupled, via the reactive cysteine on subunit III to Thiopropyl Sepharose resin (100 mg). Residual, free oxidase was removed and the resin-coupled oxidase was incubated overnight at room temperature under conditions leading to the dissociation of subunit III. Following the incubation period the resin was removed by centrifugation, the oxidase in the supernatant was recovered and found to be devoid of subunit III.

2.1. Steady-state measurements

Steady-state assays of cytochrome *c* oxidase were done using a Hewlett-Packard diode array spectrophotometer. These assays began with oxidized cytochrome *c* oxidase (~3 μM) with one equivalent of horse heart cytochrome *c* in 10 mM phosphate buffer, pH 7.0, containing 0.5 mg/mL lauryl maltoside. Sodium ascorbate (5 mM) and TMPD (200 μM) were added to start the assay and spectra were recorded over time. The initial phase of the reaction was sampled at 2 spectra per s and the remainder of the time course at 1 spectrum per 10 s.

2.2. Calculation of redox states of cytochrome c oxidase in steady state

The calculation of reduced cytochrome *a* and cytochrome a_3 is based on their contributions to the reduced-oxidized absorbance at 445 nm minus 460 nm, and at 605 nm minus 630 nm. Cytochrome *a* contributes 40% of the absorbance at 445 nm minus 460 nm, and 80% at 605 nm minus 630 nm; cytochrome a_3 contributes 60% of the difference at 445 nm minus 460 nm, and 20% at 605 nm minus 630 nm [24]. The level of reduced cytochrome *a* and cytochrome a_3 can be obtained from the following formula:

$$\Delta Abs_{445-460} = 40\%\varepsilon_1[a] + 60\%\varepsilon_1[a_3]$$

$$\Delta Abs_{605-630} = 80\%\varepsilon_2[a] + 20\%\varepsilon_2[a_3]$$

ε_1 is the extinction coefficient of cytochrome *c* oxidase at 445 nm minus 460 nm and it equals 0.156 μM^{-1} cm^{-1}; ε_2 it is the extinction coefficient of cytochrome *c* oxidase at 605 nm minus 630 nm and equals 0.027 $\mu M^{-1} cm^{-1}$; [*a*] and [a_3] are the concentration of cytochrome *a* and cytochrome a_3.

2.3. EPR spectroscopy

EPR spectra were obtained on a Bruker EMX EPR Spectrometer at X-band fitted with an HSQ cavity (Bruker Canada, Milton, ON) and liquid helium flow cryostat (Oxford Instruments, U.K.) for temperature control. The standard instrument conditions were as follows: the temperature was 10 K, modulation amplitude was 10 G at a frequency of 100 kHz and the power was 2 mW. Time-resolved absorption spectra were measured prior to transfer of the sample into an EPR tube. The sample was rapidly frozen by submersion in a bath of ethanol maintained at low temperature over a pool of liquid N_2. The frozen sample was then placed directly into liquid N_2 prior to transfer to the flow cryostat. EPR samples were taken from the different stages of the time-course: oxidized, steady state and fully reduced.

2.4. Fluorescence spectroscopy

Spectra were measured on a Spex Fluorolog-3 spectrometer. Samples were irradiated at 280 nm and emission scanned from 300 to 450 nm. The entrance and exits slits were set at a 2 nm bandpass.

2.5. Stopped-flow kinetics

The kinetics of reduction and oxidation of cytochrome *c* oxidase were measured using an OLIS rapid scanning spectrometer equipped with an OLIS stopped-flow rapid mixing device. All experiments were done in 10 mM sodium phosphate buffer pH 7.0 with one equivalent of cytochrome *c*. For reduction experiments N_2-equilibrated cytochrome *c*: cytochrome *c* oxidase (~6 μM), in the presence or absence of formamide, was mixed in an equal volume with N_2-equilibrated buffer containing 10 mM ascorbate plus 400 μM TMPD. In oxidation experiments the cytochrome *c*: cytochrome *c* oxidase (~6 μM) complex was made anaerobic, reduced with 1 mM ascorbate plus 2 μM TMPD prior to mixing with an equal volume of O_2-equilibrated buffer.

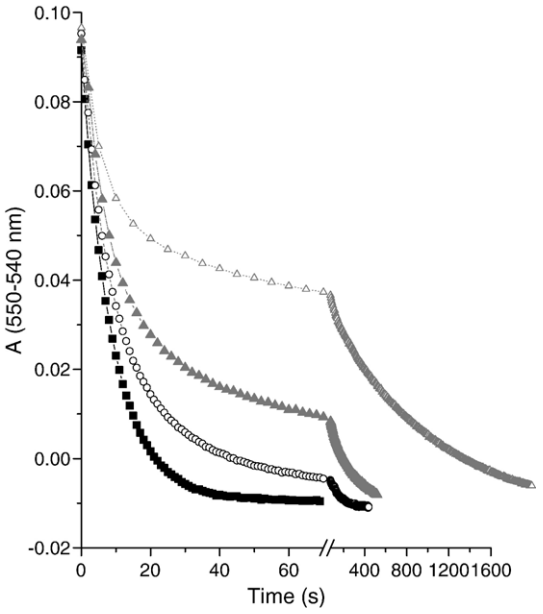

Fig. 1. The effect of formamide on the kinetics of cytochrome *c* oxidation by mitochondrial cytochrome *c* oxidase. Time courses of cytochrome *c* oxidation are measured at 550–540 nm over time at different concentrations of formamide. The concentrations of formamide used were 0 (■), 50 mM (○), 100 mM (▲) and 200 mM (8). All the runs were conducted in 50 mM sodium phosphate buffer with 0.2 mg/mL lauryl maltoside pH 7.0 and 5 nM mitochondrial cytochrome *c* oxidase. The x-axis is shown on a split time base. The lines through the data points are exponential fits to the equation, $\Delta A = A_1 e^{-kt}{}_1 + A_2 e^{-kt}{}_2$, from which the amplitudes (*A*) and rates (*k*) are determined. In the case of zero formamide the form of the oxidation fits a single exponential decay with one amplitude and rate. 0 formamide $A=0.1$, $k=0.112$ s^{-1}; 50 mM formamide $A_1=0.085$, $k_1=0.112$ s^{-1}, $A_2=0.020$, $k_2=0.0154$; 100 mM formamide $A_1=0.074$, $k_1=0.112$ s^{-1}, $A_2=0.030$, $k_2=0.0070$; 200 mM formamide $A_1=0.056$, $k_1=0.112$ s^{-1}, $A_2=0.050$, $k_2=0.0012$.

3. Results

3.1. The effect of formamide on the oxidation of cytochrome c by cytochrome c oxidase

In the absence of formamide ferrocytochrome *c* oxidation by cytochrome *c* oxidase follows a simple first order decay with respect to time [25,26]. However, in the presence of formamide the time course deviates from a first order decay and the deviation becomes more apparent as the formamide concentration is increased (Fig. 1). In the presence of formamide time courses of cytochrome *c* oxidation have two components and are fit by a double-exponential decay in which the rate of the first phase is close to that observed in the absence of formamide, whereas the second phase slows in rate and grows in magnitude as the formamide concentration increases. This pattern is a consequence of the slow-onset, turnover-dependent inhibition that has been reported for formamide [19]. When the second phase is used as a measure of the inhibited turnover and velocity is plotted versus cytochrome *c* concentration, in the absence and presence of inhibitor, formamide exhibits non-competitive inhibition (Fig. 2). The maximal turnover is diminished without affecting the affinity for cytochrome *c*. In the example shown here the maximal turnover achieved in the absence of formamide is 296 s^{-1} with a K_M for cytochrome *c* of 18.1 μM, whereas in the presence of 50 mM formamide the maximal turnover is 12.4 s^{-1} with a K_M of 14.8 μM.

3.2. Catalytic turnover of the mammalian cytochrome c: cytochrome aa_3 complex

In order to characterize the form of the enzyme that is populated in the steady state during inhibition by formamide the spectral features of the oxidase following initiation of turnover were measured. These experiments were performed at low ionic strength in the presence of one equivalent of cytochrome *c*. At low ionic strength, mitochondrial cytochrome *c* oxidase binds one equivalent of cytochrome *c* tightly to form the cytochrome *c*: cytochrome aa_3 complex. Gel filtration chromatography of mixtures of cytochrome *c* and cytochrome *c* oxidase shows that this interaction is unchanged in the presence of up to 200 mM formamide. In addition, we find that 200 mM formamide does not alter the rate of cytochrome *c* reduction by ascorbate alone, or by ascorbate plus TMPD.

Cytochrome *c*, as the natural substrate of the oxidase, accelerates electron transfer from electron donors (i.e., ascorbate plus TMPD) to the enzyme. The form of the steady-state time courses

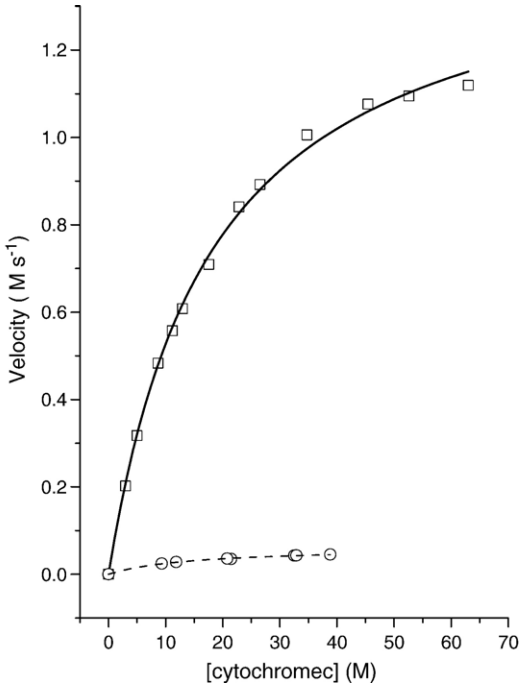

Fig. 2. Steady-state kinetic analysis of formamide inhibition of cytochrome *c* oxidase activity. The reaction velocity is plotted versus substrate concentration at 0 (□) and 50 mM (○) formamide. The lines through the data are fits to rectangular hyperbolic expression assuming a single binding site for cytochrome *c*; $v = V_{max}\,S/(K_M + S)$, where V_{max} is the maximal velocity in μM cytochrome *c*/*s*, *S* is the cytochrome *c* concentration and K_M is the affinity for cytochrome *c*. In the absence of formamide V_{max} is found to 1.48 μM/s, which corresponds to a maximal turnover of 296 s^{-1}, and K_M is 18.1 μM. In the presence of 50 mM formamide V_{max} is reduced to 0.062 μM/s and K_M is 14.8 μM. The other conditions were the same as those given in the legend to Fig. 1.

in the absence of formamide and in the presence of 200 mM formamide are shown at two wavelength pairs (Fig. 3). The redox levels of cytochrome *a* and cytochrome a_3 are measured at 445–460 nm (Fig. 3A), whereas 550–540 nm is specific for cytochrome *c* (Fig. 3B). Reduction is initiated by adding ascorbate and TMPD to the oxidized cytochrome *c*: cytochrome aa_3 complex in aerated buffer, at the time indicated by the arrows on the figure. In the absence of formamide the cytochrome *c*: cytochrome aa_3 complex is rapidly and partially reduced, and catalytic turnover ensues. Following the initial reduction of the oxidase during the turnover phase, the partly reduced enzyme relaxes to a lower reduction level than is achieved initially. This change reflects a transition of the oxidase from a less active, "resting" state to a more active, or "pulsed" state that is proposed to arise from a change in conformation of the enzyme [1,27]. After all of the dissolved oxygen is consumed, the enzyme proceeds to the fully reduced state. In this example, the enzyme was in the steady state for 75 s, corresponding to a turnover of 5.90 s^{-1}, before entering anaerobiosis. Following anaerobiosis, the enzyme proceeds to the fully reduced state with a half time of 7 s.

In the presence of 200 mM formamide, the cytochrome *c*: cytochrome aa_3 complex is rapidly reduced upon addition of ascorbate plus TMPD. However in the presence of formamide and following the initial rapid reduction of the enzyme, there is a slow transition to a higher level of reduction where the enzyme remains in an extended steady state phase (Fig. 3A). In the example shown here the steady state phase lasts for 325 s before anaerobiosis is established. The length of the steady state phase in the presence of formamide is a result of both the inhibition by formamide and the slow onset that requires turnover prior to the establishment of inhibition. Under the conditions here the enzyme only turns over about 120 times on average and much of the O_2 is consumed before full inhibition is established. The reduction process following anaerobiosis in 200 mM formamide has an observed half-time of 675 s corresponding to almost 100-fold inhibition relative to the rate in the absence of formamide (*vide supra*). In contrast, transient kinetic studies on the anaerobic reduction of oxidized cytochrome *c*: cytochrome *c* oxidase (data not shown) show that neither the rate of the initial reduction, nor the slower phase leading to full reduction is affected by the presence of formamide.

Fig. 3B follows the redox state of cytochrome *c* at 550–540 nm over the time course of the steady state reaction. In the

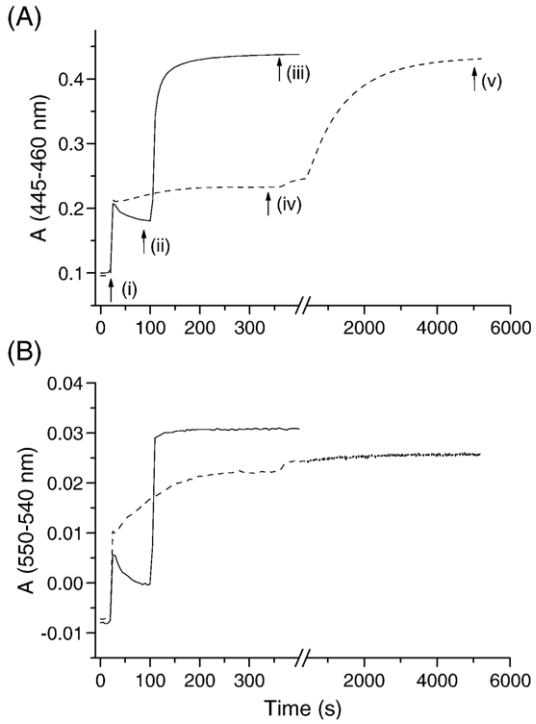

Fig. 3. Steady-state time courses of the mitochondrial cytochrome *c*: cytochrome *c* oxidase complex in the absence and presence of formamide. (A) Absorbance recorded at 445–460 nm versus time. The solid line is in the absence of formamide and the dashed line is in the presence of 200 mM formamide. (B) Absorbance at 550–540 nm versus time. In the absence of formamide, the concentration of the oxidase was 2.17 μM and cytochrome c was 1.85 μM. In the presence of formamide, the oxidase was 2.12 μM and cytochrome c was 1.70 μM. The proteins were dissolved in 10 mM sodium phosphate buffer pH 7.0 with 0.2 mg/mL lauryl maltoside. Each time course begins with the cytochrome complex fully oxidized at time zero and the reaction is started by the addition of 5 mM sodium ascorbate plus 200 μM TMPD. The series of arrows along the curves in panel A refer to the points at which the spectra used in Fig. 4 were taken: (i) fully oxidized enzyme, just before the addition of reductant, (ii) the steady-state spectrum of uninhibited enzyme, (iii) the fully reduced spectrum of the uninhibited enzyme, (iv) the steady-state spectrum of formamide-inhibited enzyme and (v) the fully reduced spectrum of formamide-inhibited enzyme.

absence of formamide, cytochrome *c* is partly reduced upon addition of ascorbate and TMPD and then relaxes to a less reduced state as cytochrome *c* oxidase transits from "resting" to "pulsed" forms. The increased electron throughput of the pulsed enzyme is reflected in the level of cytochrome *c* reduction. In this example cytochrome *c* is reduced to a level of 35% immediately after addition of the reductants and relaxes to less than 20% reduced over the course of the steady state. In contrast, in the presence of 200 mM formamide the initial rapid reaction leads to 50% reduction of cytochrome *c* that increases to about 90% during the steady-state turnover phase. Under these conditions transition to the fully inhibited, formamide state occurs with a half-time of 62 s. The kinetics of this transition is dependent on the formamide concentration as 117 s is required to achieve the inhibited steady state with 100 mM formamide and 180 s with 50 mM formamide. This is a manifestation of the turnover dependent nature of formamide inhibition and is analogous to the onset of biphasic kinetics observed in the cytochrome *c* oxidation kinetics observed above (Fig. 1).

Difference spectra of the cytochrome *c*: cytochrome aa_3 complex generated during steady-state turnover are used to analyze the form of the enzyme in the absence and presence of formamide. Fig. 4A shows the spectrum of the fully reduced minus oxidized cytochrome *c*: cytochrome aa_3 complex compared to the spectrum generated in the steady state following the resting to pulsed transition. The reduced minus oxidized spectrum is dominated by positive peaks at 445 nm and 605 nm that are characteristic of fully reduced cytochromes *a* and a_3, whereas the peak at 550 nm is due to reduced cytochrome *c*. The spectrum recorded during the steady-state phase without formamide shows partial reduction for these three cytochrome species. Assuming that cytochrome *a* contributes 40% to the absorbance at 445–460 nm, and 80% to the absorbance at 605–630 nm, that cytochrome a_3 contributes 60% at 445–460 nm and 20% at 605–630 nm and that the absorbance at 550–540 nm is entirely from cytochrome *c*, the extent of reduction of these centers in the steady state can be estimated (see Materials and Methods). Cytochrome *a* is 63.5% reduced, while cytochrome a_3 is fully oxidized, and cytochrome *c* is 20% reduced in the steady-state phase.

The spectrum of the fully reduced minus oxidized state in the presence of formamide is compared to the spectrum obtained in the inhibited steady state minus oxidized (Fig. 4B). The steady-state spectrum in the presence of formamide has strongly developed peaks at 550 nm and 605 nm relative to the fully reduced spectrum, whereas the band at 445 nm is only partially formed. The extent of reduction of cytochromes *a*, a_3 and *c* during the steady state in the presence of formamide is calculated to be 100%, 0% and 90%, respectively.

In order to view the spectral properties of the species remaining oxidized during turnover the steady-state spectrum is subtracted from the spectrum of the fully reduced species (Fig. 4C). The spectrum of fully reduced cytochrome *c*: cytochrome aa_3 minus the steady-state species in the absence of formamide is dominated by the features of cytochrome *a* and cytochrome *c* that remain significantly oxidized in the steady state. However, in the presence of formamide the steady-state contribution of oxidized cytochrome *c* is almost completely absent. The diffe-

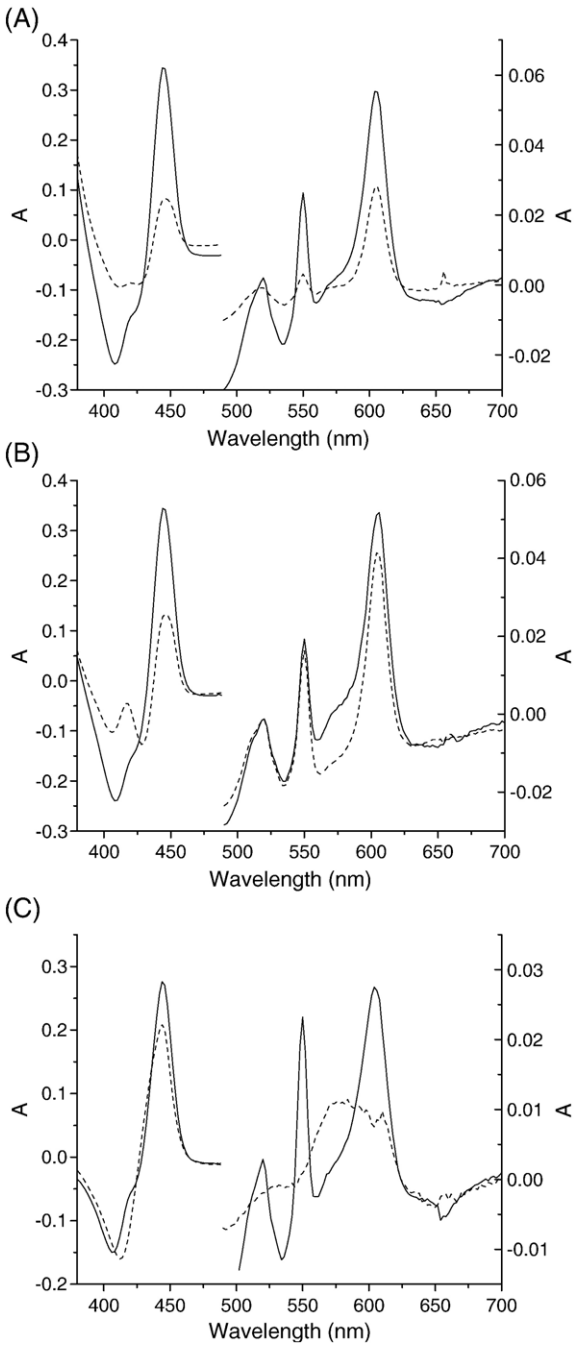

Fig. 4. Difference spectra of the cytochrome *c*: cytochrome *c* oxidase complex obtained during steady-state turnover in the absence and presence of formamide. The conditions are the same as those outlined in the legend to Fig. 3. (A) These spectra were recorded during turnover in the absence of formamide. The solid line is the spectrum of the fully reduced species obtained following anaerobiosis. The dashed line is the spectrum of the steady-state species following the resting to pulsed conversion. In both spectra the oxidized state has been subtracted as reference. (B) These spectra were recorded during turnover in the presence of 200 mM formamide. The solid line is fully reduced minus oxidized. The dashed line is formamide inhibited minus oxidized. (C) Compares the steady-state species in the absence and presence of formamide. The solid line is the fully reduced state minus the steady state in the absence of formamide. The dashed line is fully reduced minus the steady state in the presence of 200 mM formamide.

rence spectrum in the presence of formamide has a strong peak at 444 nm with a trough at 412 nm and a broad band in the visible region with peaks at 580 nm and 610 nm. These features are characteristic for the absorbance of cytochrome a_3, in a penta-coordinate high-spin state [24,28], that has remained oxidized in the presence of formamide during steady-state turnover. Such data supports the proposal that formamide inhibits cytochrome *c* oxidase turnover by slowing the apparent rate of electron transfer between cytochrome *a* and cytochrome a_3. Formamide does not appear to interfere with the progress of cytochrome *c* oxidase through any of its recognized intermediate states (e.g. Intermediates P or F) because there is no accumulation of these species in the inhibited steady state.

3.3. EPR spectra of oxidized, steady state and fully reduced cytochrome c oxidase

To further examine the spectral effects of formamide on the metal centers of the oxidase and the form of the oxidase during the steady state in the presence of formamide EPR spectra of oxidized, steady-state reduced and fully reduced cytochrome *c* oxidase were measured in the absence and presence of 200 mM formamide. Due to the higher concentration of enzyme required for EPR the time in the steady state in the presence of cytochrome *c* is very short. Therefore, in order to capture the steady state of the enzyme, cytochrome *c* was not included in the samples used for EPR. The enzyme was brought into the steady state by addition of 200 μM TMPD and 5 mM sodium ascorbate to an aerated solution in a 2 mm cuvette. At appropriate times samples were removed, placed in an EPR tube and rapidly frozen in a cold ethanol bath. The EPR spectrum of the oxidized enzyme shows no change in the intensity or position of the signals for either cytochrome *a* (g_z=3.0, g_y=2.24, g_z=1.50) or Cu_A (g_z=2.14 and g_y=2.04) in the presence of formamide (Fig. 5). In addition the intensity of the low field line centered at g=12, and assigned to the oxidized binuclear cytochrome a_3-Cu_B center, is not altered by formamide. EPR spectra during the steady state of cytochrome *c* oxidase activity in the absence of formamide show signals from oxidized cytochrome *a* (10% oxidized) and Cu_A (33% oxidized) (Fig. 5A). However, in the presence of formamide these signals disappear (Fig. 5B) indicating that both the cytochrome *a* and Cu_A centers are fully reduced in the steady state in the presence of formamide. This result is in general agreement for cytochrome *a* with the steady state UV-Vis absorption spectra. In addition there is no evidence of a new signal in the steady state in the presence of formamide from oxidized cytochrome a_3, as would be expected if Cu_B is reduced. The broad line centered at 1700 gauss in the presence of formamide is a baseline artifact. In the fully reduced sample, in the absence or presence of formamide, the peaks for cytochrome *a* and Cu_A disappear.

3.4. Effect of formamide on the intrinsic fluorescence of cytochrome c oxidase

At the concentrations of formamide used here the bulk properties of water (e.g., viscosity and dielectric constant) are

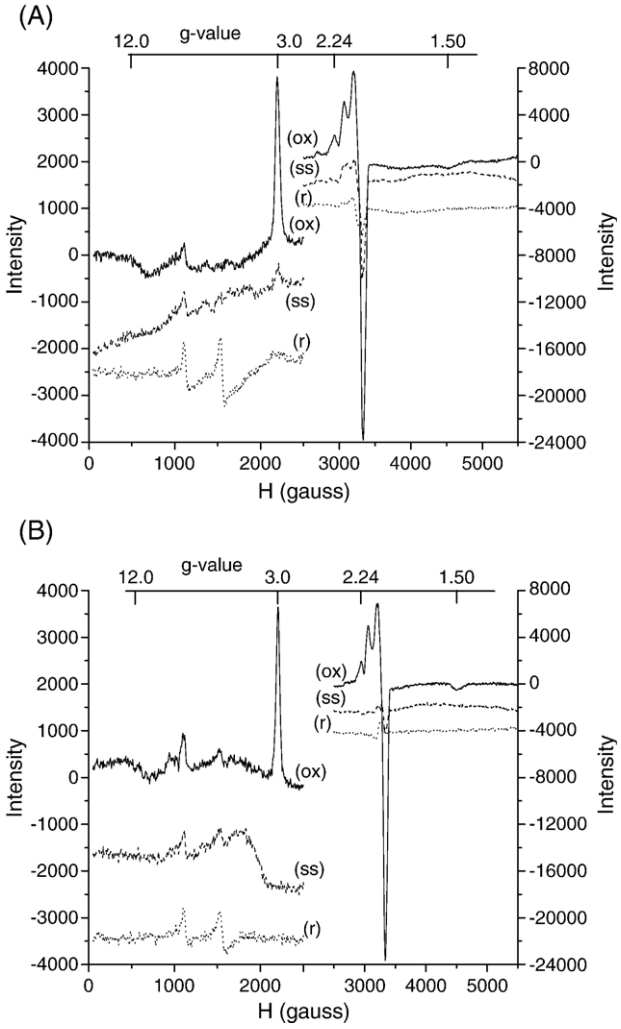

Fig. 5. Formamide effects on EPR spectra of beef heart cytochrome *c* oxidase. The concentration of beef heart cytochrome *c* oxidase was 30 μM and the EPR samples were obtained from the steady-state time-course of cytochrome *c* oxidase. The concentrations of ascorbate and TMPD were 5 mM and 200 μM, respectively. The spectrum of the buffer has been subtracted from all spectra of the oxidase. Spectra have been offset along the *y*-axis for clearer distinction. (A) In the absence of formamide, EPR spectra were recorded for oxidized cytochrome *c* oxidase (solid line, (ox)), steady-state (dashed line, (ss)), and reduced enzyme (dotted line, (r)). (B) In the presence of 200 mM formamide, EPR spectra were recorded for oxidized cytochrome *c* oxidase (solid (ox)), steady-state (dashed, (ss)), and reduced enzyme (dotted, (r)). The buffer was 10 mM sodium phosphate pH 7.0 with 0.2 mg/ml lauryl maltoside.

affected and this could lead to non-specific conformational changes in the protein that could cause loss of activity [29]. Interference from formamide in the short UV region prevents assessing the overall fold of the oxidase by UV-CD spectroscopy. However, intrinsic fluorescence of the oxidase complex can be used as a measure of the effect of formamide on the conformational state of the oxidase. The intrinsic fluorescence spectrum of the native oxidase in the absence of formamide has a wavelength maximum at 329 nm indicating that on average the environment of the tryptophan residues in the complex are shielded from water [30]. Any opening up of the structure that would allow water greater access to the tryptophan residues would cause a red shift in the emission spectrum. When the

oxidase is placed in buffer containing 200 mM formamide the emission maximum remains at 329 nm with unchanged intensity. We conclude that up to 200 mM formamide does not cause a change in the overall conformation of the oxidase.

3.5. The effect of formamide on the single turnover reaction of reduced cytochrome c oxidase with oxygen

Both EPR and UV-Vis spectroscopy support the proposal that formamide inhibition results in a slow down of electron transfer from cytochrome *a* to the cytochrome a_3-Cu_B binuclear center. This slow electron transfer results in the accumulation of reduced cytochrome *a*, Cu_A and eventually reduced cytochrome *c* in the steady state during catalysis. However, when reduced cytochrome *c* oxidase is mixed with oxygen under single turnover conditions the enzyme transits from fully reduced to fully oxidized in a few milliseconds, in the presence or absence of formamide. Thus, it appears as though formamide does not directly inhibit the rate of electron exchange between the two cytochromes, but must be involved in a redox silent step of the catalytic cycle.

3.6. The effect of formamide on the steady-state activity of B. subtilis cytochrome caa_3

The aerobic bacterium *B. subtilis* expresses a cytochrome *c* oxidase in which a cytochrome *c* domain is made as a covalent component of its subunit II [22]. *B. subtilis* cytochrome caa_3 functions in an analogous fashion to the mitochondrial cytochrome *c*: cytochrome *c* oxidase complex that is stabilized electrostatically [31]. We have tested the effect of formamide on *B. subtilis* cytochrome *c* oxidase to see if the formamide inhibition observed for mitochondrial cytochrome *c* oxidase is a feature of other enzymes of the heme-copper family. The *B. subtilis* cytochrome caa_3 complex, in contrast to the mammalian mitochondrial oxidase, goes directly into the steady state in the absence of formamide (Fig. 6A), and does not appear to undergo the resting to pulsed conversion observed for the mammalian oxidase. In the presence of formamide the onset of inhibition of *B. subtilis* cytochrome caa_3 and attainment of an inhibited, stable steady state is time dependent. In the presence of 20 mM formamide the half time for attaining an inhibited stable steady state is 8.5 s and with 100 mM formamide the half-time for full inhibition to develop is 1.5 s (Fig. 6B). Therefore, as with the mitochondrial oxidase, the onset of inhibition is turnover dependent but the development of full inhibition is almost 100-fold faster with the bacterial oxidase. The time spent in the inhibited steady state is also formamide concentration dependent. In the example shown in Fig. 6 the uninhibited steady state lasted 35 s with 0 formamide, 170 s with 20 mM formamide and 530 s with 100 mM formamide. The half time for reduction, following anaerobiosis, is 2.5 s over the initial fast phase in the absence of formamide, 100 s with 20 mM formamide and 180 s with 100 mM formamide (Fig. 6A).

Spectra were obtained from the steady-state time course of *B. subtilis* cytochrome caa_3 to analyze the redox state of the enzyme complex. In the absence of formamide cytochrome caa_3 attains a steady level of reduction in which the cytochrome

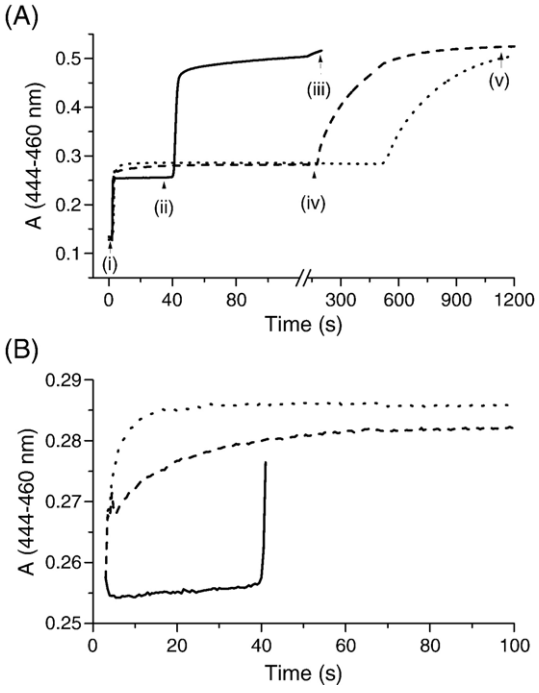

Fig. 6. *B. subtilis* cytochrome *c* oxidase activity at three different concentrations of formamide. The assay was begun by the addition of 5 mM ascorbate plus 250 μM TMPD to a solution 2.45 μM cytochrome caa_3 in 20 mM sodium phosphate pH 7.0 with 0.3 mg/mL lauryl maltoside and (——) 0 formamide, (— — —) 20 mM formamide and (– – – –) 100 mM formamide. (A) Absorbance at 444–460 nm measured as a function of time and displayed on a split time base. The series of arrows along the curves in panel A refer to the points at which the spectra used in Fig. 7 were taken: (i) fully oxidized enzyme, just before the addition of reductant, (ii) the steady-state spectrum of uninhibited enzyme, (iii) the fully reduced spectrum of the uninhibited enzyme, (iv) the steady-state spectrum of formamide-inhibited enzyme and (v) the fully reduced spectrum of formamide-inhibited enzyme. (B) Same data as in (A) displayed on an expanded *y*-axis to show the initial transition following the addition of reductant.

c and cytochrome *a* centers are about 80% reduced, with cytochrome a_3 remaining fully oxidized (Fig. 7A). In 100 mM formamide and once the enzyme has attained the steady state, the enzyme complex shows full reduction for both cytochromes *c* and *a* centers (Fig. 7B). The difference spectrum of fully reduced minus the spectrum of the steady-state species in the absence of formamide is dominated by contributions from cytochromes *c* and *a* that have remained oxidized in the uninhibited steady state. In the presence of formamide the species remaining oxidized in the steady state has broad peaks at 580 nm and 608 nm with a sharp band at 444 nm (Fig. 7C), indicating that cytochrome a_3 has remained oxidized. It is concluded that formamide has a similar effect on the *B. subtilis* cytochrome caa_3 complex in terms of diminishing the apparent rate of electron transfer from cytochrome *a* to cytochrome a_3-Cu_B, as observed for mitochondrial cytochrome *c* oxidase.

3.7. Formamide inhibition of subunit III-depleted mitochondrial oxidase

Preparations of *B. subtilis* cytochrome *c* oxidase have only the two major protein components that are common to all mem-

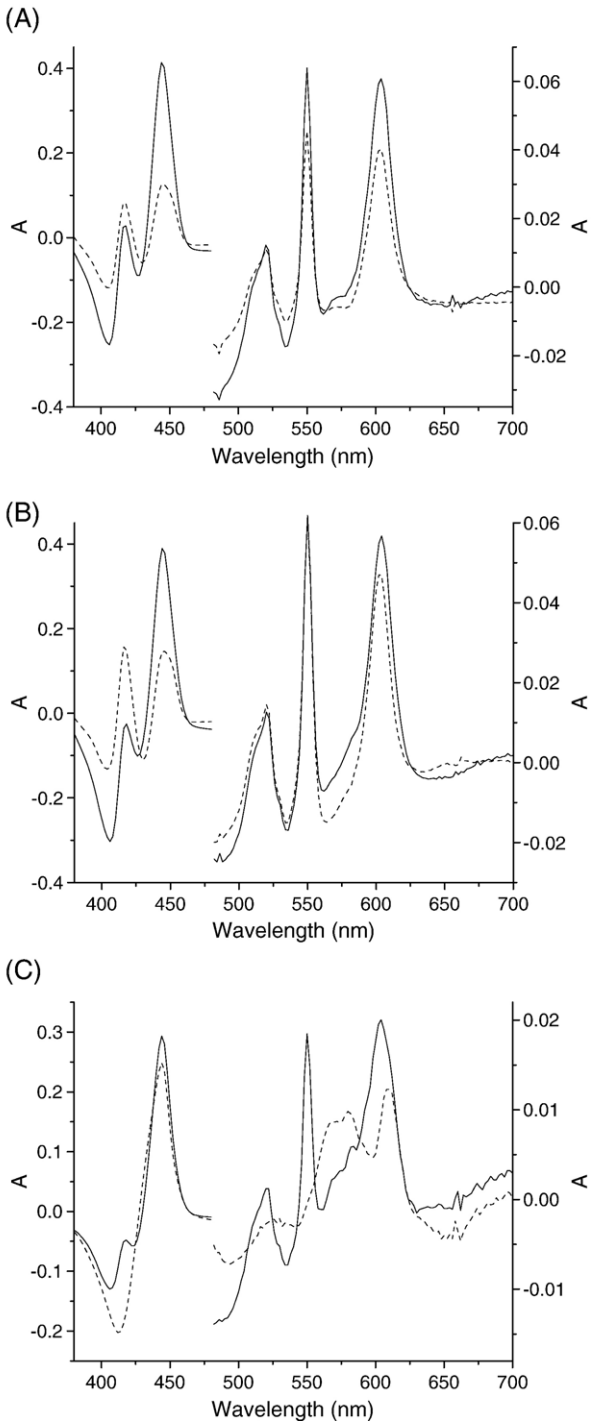

Fig. 7. Difference spectra generated during the steady-state turnover of *B. subtilis* cytochrome caa_3. The experimental conditions are the same as those given in the legend to Fig. 6. (A) These spectra are generated in the absence of formamide. The solid line (——) is the spectrum of fully reduced minus oxidized and the dashed line (— — —) is the species generated in the steady state minus oxidized. (B) These spectra are generated in the presence of 100 mM formamide. The solid line (——) is fully reduced minus oxidized and the dashed line (— — —) is the steady state minus oxidized. (C) Spectra of the species remaining oxidized during the steady state. These spectra are generated by subtracting the steady-state species from the fully reduced spectrum in (——) 0 formamide and (— — —) 100 mM formamide.

bers of the heme-copper oxidase family, subunits I and II, which contain the redox active metal centers. However, the other major conserved subunit, subunit III, and subunit IV are removed during purification of the bacterial oxidase. It has been proposed that subunit III plays a role in modulating the proton transfer pathway of the oxidase and the network of water molecules that are proposed to be involved in this process [32]. We prepared, therefore, a sample of mitochondrial oxidase from which the subunit III is removed for comparison with the holo-mitochondrial complex and the bacterial oxidase. Due to the limited number of turnovers of the oxidase in the assay conditions used here the suicide-inhibition reported for subunit III deficient oxidase [33] is not observed in our activity assay. Subunit III-depleted mitochondrial oxidase is inhibited by formamide and exhibits a time-dependent onset of inhibition as with the native protein complex. However, the onset of inhibition of the subunit III-depleted protein occurs more quickly. Subunit III-depleted oxidase reached full inhibition with 100 mM formamide with a half time of 16 s compared to 117 s with the native mitochondrial oxidase and 1.5 s with the *B. subtilis* oxidase. Access of formamide to the site of inhibition is easiest with the bacterial enzyme, but is also facilitated by removal of subunit III from the mitochondrial oxidase.

4. Discussion

4.1. Formamide does not inhibit cytochrome c oxidase via direct binding to a redox center or disruption of the protein structure

Inhibition of cytochrome *c* oxidase by azide, carbon monoxide, cyanide and nitric oxide is mediated by binding of the inhibitory ligand at the binuclear cytochrome a_3-Cu_B center. These inhibitors bind at the binuclear center to directly block the reaction with O_2, or inhibit the reduction of cytochrome a_3, which is a prerequisite for the O_2 reaction. Binding of these inhibitors to the binuclear center of cytochrome *c* oxidase elicits changes in the UV-visible absorbance spectrum, and/or the EPR spectrum. These spectral changes make it possible to investigate the thermodynamics and kinetics of the binding interactions for these inhibitors. Compared with these conventional inhibitors, formamide inhibits cytochrome *c* oxidase in a very different way. Previous studies have shown that formamide does not alter the UV, Soret, visible or copper bands of oxidized or reduced cytochrome *c* oxidase [19]. These results and those reported here support the view that formamide inhibition does not involve direct ligation to any of the redox centers as is the case with the conventional inhibitors mentioned above.

Lack of a pronounced spectral effect on any of the redox active metal centers also implies that formamide inhibition does not lead indirectly to a change in the immediate environment of the redox active centers. Formamide inhibition seems to arise via interaction with the oxidase at a site that is remote from the enzyme's redox active metal centers. Since formamide is known to disrupt the bulk properties of water, formamide could lead to a non-specific protein conformational change, or partial denaturation. However, fluorescence spectroscopy of formamide-inhib-

ited cytochrome *c* oxidase does not show any shift of the fluorescence emission peak indicating that the environment around the aromatic residues of the oxidase is largely unaltered in the presence of formamide. In addition formamide does not alter the rates of intrinsic electron exchange within the oxidase as measured in single turnover experiments. The lack of an effect of formamide on the intrinsic electron transfer rates also suggests that formamide does not effect the redox potentials of the two hemes. Therefore, it is concluded that formamide does not bind to the redox centers, or induce a drastic conformational change of the oxidase to alter electron transfer rates. It is interesting to note in this context that studies on the cytochrome *bd* oxidase from *Escherichia coli* up to 5M formamide was found to have no effect on either its UV-visible spectral properties or catalytic activity [34]. The cytochrome *bd* complex is an integral membrane enzyme that catalyzes the reduction of O_2 to water using ubiquinol as the reducing substrate. However, cytochrome *bd* is not a member of the heme-copper oxidase family and does not couple the oxidation of ubiquinol to proton pumping.

4.2. Inhibition by conformational trapping

Formamide inhibition could be the result of a more subtle conformational based effect. The oxidase is proposed to undergo conformational changes as part of its catalytic cycle and these changes serve to link the chemical reactions of electron and proton transfer [1]. Conformational trapping has been invoked to explain the effect of subunit-specific antibodies in stimulating [35] and inhibiting [36] cytochrome *c* oxidase activity. If formamide were able to stabilize a particular form of the oxidase and block the molecule's progress through its conformational cycle this would lead to a slow down of catalysis and could result in the accumulation of reduced cytochrome *a* and cytochrome *c* observed here. There is an indication that formamide blocks the turnover dependent transformation of resting to pulsed enzyme that is observed with the mitochondrial oxidase and is important for achieving maximal activity. However, the *B. subtilis* oxidase does not undergo the resting to pulsed conversion and yet formamide is still an effective inhibitor of this bacterial oxidase. We propose, therefore that formamide does not exert its inhibitory effect by trapping the oxidase in a resting conformational state.

4.3. Formamide disrupts the water network within the oxidase

Another mode of inhibition to consider for formamide is as a disruptor of the proton transfer pathway(s) of the oxidase. It is now recognized that much of the proton transfer process through the oxidase, both for pumped protons and those consumed in water formation, takes place at sites remote from the metal centers. The proposed proton pathways involve a relatively small number of amino acids directly, with water molecules making up the intervening proton conducting medium between key amino acid residues. If formamide were to enter the proton conducting water chain within the oxidase it could slow the transfer of protons due to the higher energetic barrier calculated for proton transfer via formamide as compared to water [37]. The D-cahannel begins on the surface of subunit I on the inner side of the membrane at subunit I's interface with subunit III. Our observation that the onset of formamide inhibition is faster in the absence of subunit III suggests that formamide inhibition results from disruption of the D-channel. However, an energetic mechanism for formamide inhibition alone cannot account for the turnover dependent inhibition exhibited with cytochrome *c* oxidase.

If the inhibition of formamide were also to require turnover-dependent uptake of formamide into the protein matrix in the place of water this would account for the slow onset of formamide inhibition. On this basis Kornblatt [19] proposed the existence of a water cycle as part of the catalytic cycle of the oxidase. Alternatively, this water cycle would be accommodated if H_3O^+ were an element in the proton pump of the oxidase. If formamide were taken into pumping pathway this would severely curtail the kinetics of proton flow. The apparent inhibition of electron delivery to the binuclear center and the slow down of catalytic turnover observed here would then be a function of proton-gated nature of this step in catalysis (e.g., [38]).

4.4. Formamide disrupts the proton pool available to the oxidase

Apart from the assumption that formamide needs to get into cytochrome *c* oxidase to inhibit its catalytic activity, another possible mechanism is that formamide may directly interact with the mitochondrial matrix-exposed surface of cytochrome *c* oxidase to affect access of the D- or K-pathways to bulk protons. The rapid turnover of cytochrome *c* oxidase requires an available proton pool nearby. Recent studies have pointed out that the cluster of negatively charged amino acids and six histidines in the entrance to the D-pathway may combine to form a proton-collecting antenna [39,40]. Additional to these amino acids, Asn and Gln, whose residue ends have similar structure to formamide, both distribute richly in this zone [9,13]. All these amino acids plus water may together form an effective proton pool providing protons for the enzyme. It is possible that formamide disturbs the proton-gathering antenna with its ability to be involved in hydrogen bond networks. The proton pump coupled electron transfer would thus be affected in an indirect manner. Such a mechanism for formamide inhibition would allow the single turnover reaction with O_2 of formamide-inhibited oxidase to proceed at the same rate as the uninhibited enzyme. However, subsequent turnover would be inhibited by the limited availability of protons. Although this is an intriguing possibility it does not explain the slow onset of inhibition by formamide unless the water is under slow exchange with bulk solvent and this exchange is somehow mediated by catalytic turnover.

Formamide is a remarkable solvent. It has been known as a disruptor of double helix formation between nucleic acids [41] and has been used to prevent unwanted association between nucleic acids in a variety of technical approaches (e.g., [42]. It has been proposed that the mechanism of formamide induced dissociation of nucleic acid duplexes is driven by the ability of

formamide to disrupt the hydration state of nucleic acid molecules [43]. Formamide has been considered as a possible alternative to water as a viable biological solvent under some conditions [44]. At this point we do not have direct evidence that formamide inhibition is mediated by interference with specific interactions between cytochrome *c* oxidase and water. However, we propose that formamide may prove useful as an experimental probe of proteins involved in the catalysis of water transport.

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