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The Water Channel of Cytochrome *c* Oxidase: inferences from Inhibitor Studies

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ABSTRACT Cytochrome *c* oxidase couples electron transfer to proton transfer from inside the mitochondrion to the cytosol. Protons pass through a channel; it is closed except when protons are pumped. Electron transfer is also coupled to a water cycle. Water moves into and out of the oxidase during electron transfer, presumably through a channel. The three processes are coupled because of the common dependence on electron transfer. If water and protons had to pass through the same channel for the proton to pass, it might be possible to block the pore by entraining small molecules in the flow. The data in this report indicate that there is a correlation between the ability of a compound to inhibit the oxidase and its size. Formamide and formaldehyde are potent inhibitors. Larger and smaller molecules are poor inhibitors. Formamide introduces an internal block in electron transfer. It is a slow-onset, reversible inhibitor, dependent on turnover to manifest its effects. Vesicular oxidase is less influenced by formamide than is soluble oxidase; formamide must pass a permeability barrier to act. The data are consistent with a proton channel with constrictions at both ends that open to yield a pore of ~4 Å.

INTRODUCTION

Cytochrome *c* oxidase is the terminal electron carrier of the mitochondrial electron transport chain and a site of energy coupling (see Babcock and Wikström, 1992, and Yoshikawa, 1997, for relatively recent reviews). The protein from ox heart is complex. It consists of 13 different protein subunits. There are two hemes; one is known as cytochrome *a*, the other as cytochrome a_3. There are three coppers, one as CuB and the other two as a single dinuclear copper site, CuA. The x-ray structure of the ox heart enzyme was recently solved (Tsukihara et al., 1995, 1996) and is available from the protein data bank (PDB1OCC). There is also a crystal structure of the four-subunit Paracoccus enzyme (Iwata et al., 1995) as well as the two-subunit Paracoccus enzyme (Ostermeir et al., 1997); the latter is available in the data bank (PDB1ARI). A comparison of the core structures of the two proteins shows them to be quite similar. Other terminal oxidases from bacteria and eukaryotes are thought to have very similar core structures.

As an enzyme, the protein accepts electrons one at a time from cytochrome *c* and transfers them to oxygen. The mechanism is complex. It involves transfer into the dinuclear copper center, CuA, followed by rapid equilibration with cytochrome *a*. There is probably a two-electron transfer into the cytochrome a_3/CuB center. Subsequent steps are dictated by the availability of reduced cytochrome *c* and O_2. The overall kinetics have been reviewed (Hill, 1988, 1991, 1994; Einarsdottir, 1995). In any event, two electrons are ultimately transferred out of the oxidase to form a bound peroxide intermediate, which is then fully reduced to water. As electron transfer occurs, energy is conserved in an electrochemical gradient of protons. This gradient arises from two sources: 1) protons are pumped out of the mitochondrion; 2) protons are consumed in the matrix space as a result of the reduction of oxygen. In the first process, there has to be a pore through which the protons can be passed. In the second process, protons must have access to a site close to where oxygen is reduced. Both processes require some form of phenomenological pore for function to occur.

Inspection of either the ox heart or the Paracoccus enzymes shows proteins that clearly span a membrane and have access channels leading to the region of the cytochrome a_3/CuB center from the inside of the organelle or cell. There are also access channels that lead to this same general region from the outside of the organelle or cell. The inside and outside access channels do not meet to form a continuous pore. They are blocked by ~10 Å of closely packed protein that has very small void spaces. Pumping the protons across the membrane involves passing this block.

Hydrostatic and osmotic pressure studies have indicated that for electron transfer from cytochrome *c* to oxygen to occur, the hydration state of the protein must change in a cyclical fashion (Kornblatt et al., 1988; Kornblatt and Hui Bon Hoa, 1990). Water must enter and leave the protein. Inhibiting the free flow of water inhibits electron transfer, indicating that the two are well coupled and that little slippage occurs. Dilatometry experiments have quantified some of the differences in hydration that exist between the different intermediates (Kornblatt et al., 1998). The hydration differences show up as differences in density between the intermediates. As the protein cycles, water goes from the bulk solution into a compartment where its density is different. The simplest hypothesis is that this compartment(s) is in the interior of the protein. Regardless of where the water is sequestered, regardless of whether a portion of the

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water is formed by the reduction of oxygen, the cycle is obligatory; electron transfer does not occur without it.

In its proton-pumping capacity, the oxidase also shows little slippage. Electron transfer is normally tightly coupled to proton transfer; this is the phenomenon of respiratory control. Via site-directed mutagenesis, the oxidase can be made to slip, can be made inefficient (Fetter et al., 1995), but this is not the normal state of affairs.

In sum, the oxidase as an enzyme plugged into a membrane has three cycles: electron transfer, proton transfer, and water transfer. The first and second are coupled, the first and third are coupled, and the second and third must therefore be coupled. Is the necessity for a water cycle the result of a process whereby hydrated protons are passed from one side of the membrane to the other, or is it just fortuitous? If water must pass into and through a privileged compartment as a solvent for protons, it might be possible to interfere with its function passively. It might be possible to block the water channel with compounds that are slightly larger than water; still larger molecules should be poor inhibitors. The data presented in this paper show that this is the case. The data are consistent with the view that proton transfer through the protein is accompanied by simultaneous water transfer.

MATERIALS AND METHODS

Cytochrome *c* oxidase was purified from ox heart as previously described (Yonetani, 1966). Before use Tween 80 (1 mg/mg protein) was added, and the cholate was removed by dialysis. Cytochrome *c* (prepared without trichloroacetic acid) was purchased from Sigma Chemicals. Acetamide (certified), *N,N*-dimethyl formamide (Spectranalyzed), formaldehyde (37% w/w certified A.C.S.), formamide (superpure grade), and methanol (spectranalyzed) were purchased from Fisher Chemical Co. Ethanol (95%), tetramethyl urea, and urea (Ultrapure) were purchased from ICN. Ethylene glycol (Analar) was from BDH.

The 37% formaldehyde was diluted to 10% with water. Titration of the resulting 3.3 M solution with KOH showed that it contained 1.5 mM formic acid. The purity of the formamide was checked by NMR; it was at least 99.5% pure. The other compounds were used as is and were not checked for purity.

All of the products were modeled using the program PCMODEL. The data obtained from this program did not differ substantially from those obtained with InsightII from Biosym.

Spectra of the oxidase and its inhibited complexes were recorded on a Cary 2290 UV/Vis spectrophotometer thermostatted at 15°C. Oxidase activity was measured spectrophotometrically on an Aminco DW-2 spectrophotometer or using a Yellow Springs Instrument Co. Clark-type electrode, both thermostatted at 25°C. For the Michaelis-Menten type kinetics, we used only the first two seconds of the total trace; for this we equipped the DW-2 with a stopped-flow attachment. All kinetic traces were analyzed using digitized data taken with an Enhanced Multilab Card PCL-812PG interfacing the DW-2 to a computer.

The buffer for all measurements, except those involving vesicles, contained 10 mM Tris, 10 mM EDTA, 1 mM NaF, 100 mM NaCl, and 0.5% Tween 80 (pH 7.2). There was no Tween present in the buffer for vesicle measurements.

RESULTS

Many inhibitors of cytochrome *c* oxidase inhibit the protein by binding to the pentacoordinate cytochrome a_3 heme or CuB or both. The compounds used for this study were chosen because they were, in the main, unreactive and small. This is not the first such study of the effect of small molecules on cytochrome *c* oxidase (Cabral and Love, 1974; Kornblatt and Hui Bon Hoa, 1990); previous work used ethylene glycol, glycerol, and sucrose to establish that the hemes were in an environment protected from these perturbants. Table 1 groups water along with the nine different compounds used on the basis of the concentration needed for 50% inhibition. The data for water are not meant to represent that of the bulk product. I am trying to compare molecules that are not associating with one another, so feel justified in using data that come directly from a modeling program. There are two listings for formaldehyde. In aqueous solution it exists primarily as a hydrated structure in which the ratio of hydrate to aldehyde is 2000:1 (Bell, 1966).

There are clearly only two inhibitors that are at all potent; these are formamide and formaldehyde. The other seven tend to require concentrations in excess of 1 M to bring

TABLE 1 Inhibition of cytochrome *c* oxidase by small, unreactive compounds

Inhibitor	Concentration for 50% inhibition	Estimated volume (Å^3)	Estimated longest dimension (Å)
Water		25	1.49
Ethylene glycol (8 m)	8 M	82	4.57
Methanol	4.8 M	52	2.83
Ethanol	2.2 M	76	3.55
Urea	2 M	72	3.61
Acetamide	1.75 M	81	4.04
Dimethylformamide	1.2 M	107	4.39
Tetramethyl urea (product is not pure)	>1 M	171	5.63
Formaldehyde	100 nM	45	2.03
or formal * H_2O		59	2.82
Formamide	5 mM	58	2.87

The lengths, areas, and volumes were based on the program PCModel. There are comparable data from Goldstein and Solomon (1960), which are based on average radii of Catlin models. The values are water, 1.5 Å; ethylene glycol, 2.34–2.13 Å; urea, 2.1–1.95 Å; acetamide, 2.29–2.24 Å. Sha'afi et al. (1971) measured a radius of 2.07 Å for formamide. *Note:* These data are radii, whereas the values in the table correspond to the longest dimension.

about 50% inhibition. The striking feature of the two potent inhibitors is that they have similar volumes and the longest dimensions. Although the estimated longest dimension of methanol is similar to those of formaldehyde and formamide, the unadjusted molar volume of formamide is at least 6 $Å^3$ (10%) greater than that of methanol. Furthermore, the hydrogen bonding patterns of methanol, formamide, and formaldehyde are different (Stein, 1967). Methanol will form hydrogen bonds with two partners, whereas formamide will hydrogen bond to three. The hydrated formaldehyde hydrogen bonds to four partners. The implication is that methanol is functionally smaller than the other molecules studied. The lengths and volumes of Table 1 are all based on modeling; the data presented in the table make no allowance for the known hydrogen bonding properties of the compounds, their permanent and induced dipoles, or for any other properties. The different compounds, with the exception of formaldehyde, have all been treated as though they were hard, noninteracting prolate spheres.

Formaldehyde is a difficult compound with which to work; it is contaminated with the oxidation product formic acid, itself an inhibitor of the oxidase. Titration showed that the formaldehyde contained 1 part in 2000 as formate. At this ratio there would be 0.05 mM formate in our kinetic assays, well below the K_i of ~10 mM (Nicholls, 1975). Formaldehyde chemistry is further complicated, in that it reacts with protonated primary amines liberating acid. At the concentrations used here, the formation of the Schiff base adducts with both cytochrome *c* and cytochrome *c* oxidase contributes to the overall inhibition pattern, but we cannot say to what extent. In sum, the 100 mM formaldehyde needed for 50% inhibition contains formate, formaldehyde, the hydrated adduct of formaldehyde, and the Schiff base adduct of formaldehyde. I prefer to avoid drawing any conclusions about the mechanism by which this compound inhibits, other than to say that the inhibition cannot, in the main, be due to formate.

The formamide inhibition is the most interesting. The commercial compound is unreactive and very pure and was verified as very pure before use. The remainder of the data presented in this article are concerned with establishing a mechanism by which it inhibits the oxidase.

Fig. 1, *A* and B, shows the effects of formamide on the spectra of cytochrome *c* oxidase. Formamide had no detectable effect on the UV (not shown), Soret, visible, or copper bands (not shown) of the oxidized protein (Fig. 1 *A*). Fig. 1 *B* shows that adding reductants to the oxidase/formamide mixture brought about complete reduction of the oxidase. The sample containing 200 mM formamide (*dotted trace*) required more than 2 h to be fully reduced, whereas in the absence of formamide the reduction took seconds (not shown). The same inhibitory pattern was seen with either cytochrome *c* as the reductant or TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine) as the reductant. There was no observable difference between the fully reduced oxidase that contained formamide and the fully reduced enzyme that lacked it. On the other hand, before the oxidase/formamide

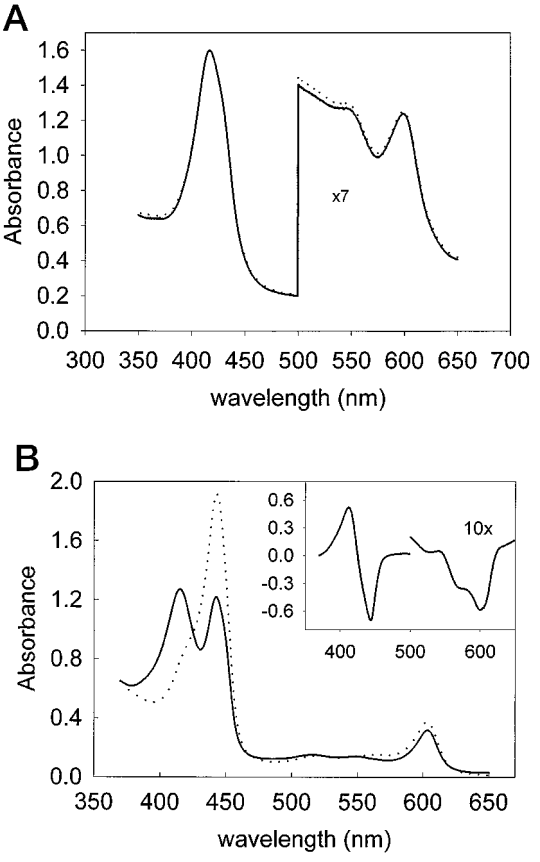

FIGURE 1 Formamide has no measurable effect on the spectra of cytochrome *c* oxidase but does introduce a block in internal electron transfer. (*A*) The complete system contains 100 μM oxidase in a 1-mm cuvette. ——, The spectrum without formamide; ·····, the spectrum with 0.2 M formamide. There are no significant differences between the two spectra in the UV (not shown), the Soret region, the 600-nm visible region, or in the 830-nm CuA region (not shown). (*B*) Cytochrome *c* (1 μM) and ascorbate (100 mM) were added to the formamide-containing sample of *A*. The cuvette was covered with a layer of mineral oil, and a spectrum was taken (——). It took 2 h instead of seconds for the characteristic reduced spectrum (·····) to appear. (*Inset*) The difference spectrum (approach to reduced (——) minus reduced (·····)).

mixture was fully reduced, the inhibitor introduced a block in electron transfer between cytochrome *a* and cytochrome a_3. The split in the two heme bands (*solid trace*), seen so clearly in the region between 410 nm and 450 nm and emphasized in the difference spectrum (Fig. 1 *B*, *inset*), is indicative of a block in internal electron transfer. A similar block in the internal transfer from cyt *a* to cyt a_3/CuB is introduced by both high osmotic and hydrostatic pressure, indicating that we are at least dealing with the same locus in these very different kinds of experiments. The two pressure perturbants act at the level of hydration of the oxidase.

Inhibition of cytochrome *c* oxidase activity is dependent on turnover. There is little inhibition after only a few turnovers; there is substantial inhibition after many turnovers. Table 2 compares the effect of 500 mM formamide on oxidase activity during the first 2 s of turnover. The data were taken using a stopped flow as a rapid mixing device.

TABLE 2 Effect of formamide on the short-term kinetics of cytochrome *c* oxidase

Kinetic constant	No formamide in assay	+500 mM formamide
K_m	11.0 ± 0.4 μM	9.4 ± 2.1 μM
Turnover number (e^-/s)	213 ± 5 e^-/s	153 ± 8 e^-/s

The data were evaluated during the first 2 s of a stopped-flow trace. The data were evaluated under the assumption that they would fit a standard Michaelis-Menten kinetic plot. At the ionic strengths used here, ~150 mM, the assumption is reasonable. Five concentrations of cytochrome *c* were used in each analysis, and each velocity point was the average of 10 shots with the stopped flow. Each K_m and turnover number were evaluated with the program ENZFITTER by Leatherbarrow. The standard errors shown were obtained from the same program.

The oxidase itself had been dissolved in the formamide-containing solution and had been incubated for at least 1 h with the formamide before a kinetic run was started. The data are quite clear. During the first few turnovers of the protein, formamide has little effect on oxidase activity. Inspection of the two progress curves in Fig. 2 shows that catalytic activity, when formamide is present (*upper curve*), is inhibited as the reaction proceeds: formamide is a slow-onset inhibitor that is dependent on turnover to express its inhibitory potential.

The inhibition of the oxidase by formamide is almost complete at high concentrations of the latter. As one lowers the concentration of formamide, it takes longer for "complete" inhibition to set in and more turnovers are required. There is always a slow "leak," less than 1% of maximum oxidase activity at the highest concentrations of formamide. The leak makes it difficult to estimate the exact time that full inhibition is established. It is a somewhat easier matter to establish how many cytochrome *c* are oxidized as a function of formamide concentration. One extrapolates the steady-state leak back to the point of oxidase addition; this results in an underestimation of the number of cytochrome *c* required for complete inhibition, but the error is not serious. These data are shown in Table 3. One can see that the number of turnovers per oxidase (column 4) increases by 14 as the formamide concentration is decreased by a factor of 24. In each instance inhibition was almost total. This means that the ratio of formamide to turnovers remains approximately constant and, by extension, that the number of waters per turnover remains approximately constant (see legend to Table 3). I postulate here that formamide is inhibiting by a purely stochastic process in which it is mistaken for water. Furthermore, having been mistaken, formamide blocks a water channel. Accordingly, 3.40 ± 0.38 waters would have to move through the oxidase for every $4e^-$ turnover (column 5). I return to this point later because it is one of the two important points to be made by this work.

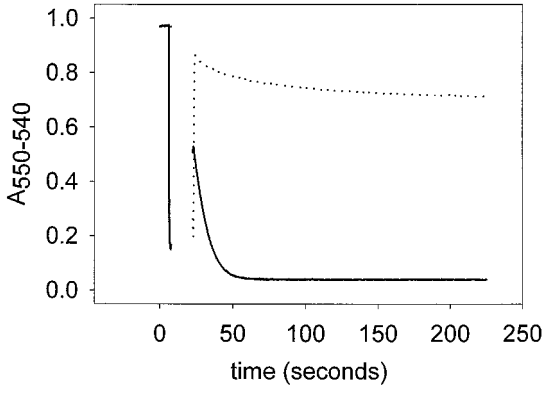

FIGURE 2 The inhibition of oxidase activity by formamide. The 3-ml cuvette contained 50 μM cytochrome *c* with or without 303 mM formamide. The reaction was started by adding 50 μl of 6 μM oxidase. The earliest portion of the trace is too fast to be seen. The control sample (*lower trace*), containing no formamide, goes to zero over the course of the 25 s from the addition of oxidase. Oxidase was added ~5 s before the start of the downward traces.

The inhibition of oxidase activity by formamide is totally reversible. Fig. 3 shows that this occurs with a rate constant of $\sim 2.5 \times 10^{-3}$ s^{-1} or a half-time of 274 s. For this experiment, oxidase at 20 μM was allowed to turn over in the presence of 209 mM formamide and then diluted 1:1000 into fresh buffer, omitting the formamide and additional reductants (the diluted sample still contains 1 nM cytochrome *c* and 100 μM ascorbate). The diluted material was assayed at the times indicated. The recovery follows a simple first-order exponential. There is no apparent lag in the recovery process. The lack of a lag and the simple exponential decay indicate that the assumption of one formamide needed to inhibit one oxidase (see Table 3) was reasonable.

TABLE 3 The number of cytochrome *c* oxidized per oxidase, before the onset of "complete" inhibition, is a function of the formamide concentration

Formamide (mmolal)	Oxidase (nM)	cyt c^2 oxidized (μM)	Cyt *c* per oxidase	H_2O per turnover*
486	128	14.3	112	3.06
486	128	14.3	112	3.06
406	130	18.5	142	2.84
303	133	21.6	162	3.25
203	135	28.4	210	3.7
102	70.1	27	385	3.9
86.3	56.4	27.2	482	3.67
67.1	28.5	20.2	708	3.18
40.7	21.5	21	976	3.8
20.7	14.4	22.6	1570	3.55

*The number of water molecules per turnover was calculated as follows:

$$H_2O/\text{turnover} = (H_2O/\text{Formamide})/(\text{Turnovers/Formamide})$$

$$H_2O/\text{turnover} = (H_2O/\text{Formamide})/(((\text{cyt } c^2)\text{ consumed})/(aa_3))/(1\text{ formamide})$$

(H_2O/Formamide) is obtained directly from themolality of the solution.

(((cyt c^2) consumed)/(aa_3))/(1 formamide) is the total change in cyt c^2 after addition of oxidase to the formamide-containing reaction mixture. The assumption in the calculation is that one and only one formamide is required for the complete inhibition of one oxidase molecule.

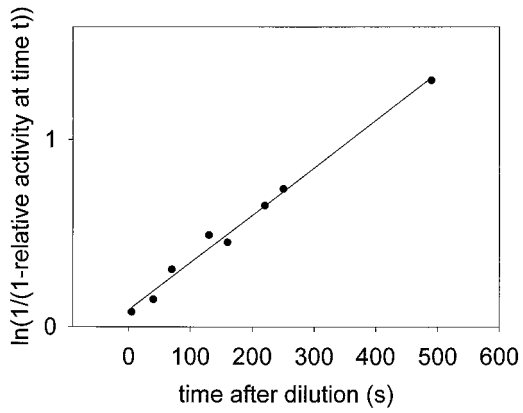

FIGURE 3 The inhibition of cytochrome *c* oxidase by formamide is completely reversible. Oxidase (40 μM), containing 209 mM formamide, 1 μM cytochrome *c*, and 100 mM ascorbate, was incubated until inhibition was complete. Aliquots were diluted 1:1000 into fresh buffer, and the rate of recovery of activity was determined at the times shown. The recovery rate is 2.53×10^{-3} s^{-1}.

What concentration of formamide is required for 50% inhibition of oxidase activity, and how does this concentration relate to the above recovery constant? Table 1 indicates that 5 mM formamide is required for 50% inhibition of oxidase activity. Fig. 4 shows the results of an experiment in which 1.19 nM oxidase was assayed in the presence and absence of 5 mM formamide. At this concentration, the ratio of water to formamide is 11,500:1. On average the oxidase had to turn over 11,500 electrons before the rate of oxidation dropped to and maintained 50% of the control value.

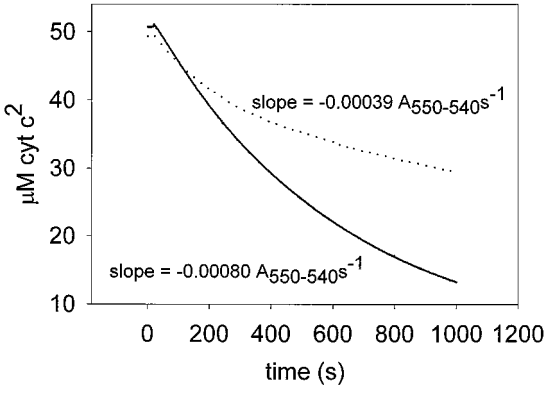

FIGURE 4 The inhibition of cytochrome *c* oxidase activity by 5 mM formamide. The ratio of water to formamide was 11,500:1. Each oxidase had to turn over 11,500 electrons before it saw, on average, a single formamide. The concentration of oxidase was 1.19 nM, and the concentration of reduced cytochrome *c* was 50 μM. The oxidase was required to consume 13.7 μM cytochrome *c* (11,500 $\times$ 0.00119 μM) before it saw, on average, one formamide. The slopes were the same at the start of the experiment. The slope when the 13.7 μM had been consumed was $-0.00039A_{550-540}$ s^{-1} in the presence of formamide and $0.00080A_{550-540}$ s^{-1} in the absence of formamide. The ratio of the slopes is close to 0.5; this means that on average, the presence of one formamide is sufficient to block 50% of the electron transfer. The recovery rate from Fig. 3 is 2.5×10^{-3} s^{-1}. The rate constant for onset and maintenance of inhibition under these conditions is 1.4×10^{-3} formamide/s/oxidase.

The turnover rate of the inhibited oxidase under these conditions was 15.4/e^-/s/oxidase. Because the level of inhibition remains constant, the rate at which formamide molecules enter the channel must equal the rate at which they leave the channel. This can be expressed as

$$\text{Oxidase} + \text{Formamide} \xrightarrow[\text{In}]{k_1}$$

$$\text{Oxidase/Formamide} \xrightarrow[\text{Out}]{k_2} \text{Oxidase} + \text{Formamide}$$

The rate of entry can be calculated from the turnover rate by assuming that formamide enters via a process in which it is mistaken for water. The data of Table 3 indicate that 0.85 waters enter per electron. At 5 mM formamide the ratio of water to formamide is 11,500. The rate of entry of formamide then equals

$$(15.4\ e^-/\text{s/oxidase}) \times (0.85\ \text{water}/e^-)/(11{,}500\ \text{waters/formamide}) = 1.14 \times 10^{-3}\ \text{formamide/s/oxidase}$$

The value differs from the recovery constant by a factor of 2. The similarity between the two numbers is good, considering the nature of the experiments.

The effects of formamide on the activity of coupled vesicular oxidase are interesting and critical to the postulate that the water and proton channels are intimately related. Coupled oxidase vesicles are closed, hollow spheres of phospholipid through which the oxidase is plugged; the outer diameter of these spheres is variable, but the majority are between 20 nm and 50 nm (Tihova et al., 1993; Kornblatt, unpublished observation). The majority of the oxidase molecules have their cytochrome *c* binding sites exposed (Kornblatt et al., 1975). The major characteristic of these vesicles is that they develop an electrochemical gradient of protons across the membrane, and this gradient slows the rate of electron transfer from cytochrome *c* to oxygen. Protons are pumped from the internal space of the vesicle to the outer bulk solution; the phospholipid bilayer provides the permeability barrier to the protons. The gradient can be collapsed with the classical uncouplers carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin. The former collapses the proton gradient, whereas the latter acts to collapse the membrane potential. If formamide acts by blocking a channel, and if that channel is part of a proton pump that functions in the outward direction, then the inhibitor should be less effective on vesicles than it is on soluble oxidase, where the pump is readily accessible to the bulk solution; the phospholipid bilayer would, in this instance, provide a barrier to the free diffusion of formamide to the blocking site. If the speculations about formamide acting at the level of the proton channel have any validity, formamide would have to diffuse across the lipid bilayer for it to inhibit. This diffusion would be more difficult for formamide than it would be for water itself; Finkelstein

(1976) showed that the permeability coefficient of formamide through a lipid bilayer was ~1/20 to ~1/30 that of water and that the ratio was more or less independent of the nature of the phospholipid.

Table 4 indicates that formamide is a less effective inhibitor of vesicular oxidase than it is of soluble oxidase. In the coupled state, where the oxidase is turning over slowly, the inhibitor is ~76% as effective on the vesicular oxidase as it is on the soluble oxidase. When the rate of electron transfer is increased through the addition of FCCP and valinomycin, the inhibitor is only ~60% as effective. These data indicate that for formamide to inhibit vesicular cytochrome *c* oxidase, in contrast to the soluble enzyme, formamide must pass a diffusion barrier. Would many formamide molecules have to be involved for this diminution of inhibitory capacity to occur? At 50-nm external diameter (~40 nm internal) and 5 mM formamide, the total internal space would contain only ~100 molecules of formamide. Reducing the number of formamide by 20 molecules would more than account for the difference in rates. Although calculations such as these are not meaningful, they illustrate the nature of the problem. There is a limited space available inside the vesicle; whether the inhibitor has access to the site of inhibition is not easily dealt with by using standard solution kinetics. The one thing that can be stated unequivocally is that the inhibition site is not as accessible in vesicular oxidase as it is in soluble oxidase.

DISCUSSION

Mitchell's original formulation of the chemiosmotic hypothesis was based on intuition derived from physiology and thermodynamics (Mitchell, 1979). The view was that an electron transfer protein could harvest the energy associated with electron transfer if it could store that energy in the form of a gradient. The electron transfer protein would effectively establish an electrochemical cell in which protonic activity was different on two sides of a membrane. The protein, asymmetrically plugged into the membrane, would have acted as one of a pair of transducers. The other member of the pair would be another protein that could use the resulting electrochemical gradient of protons as a functional energy source to synthesize ATP. Wikström and his colleagues showed in a long series of truly elegant experiments that the terminal electron transfer protein, the cytochrome *c* oxidase, was not a passive player in this establishment of the proton gradient, but rather acted as a proton pump (Wikström, 1977; Wikström and Krab, 1978). The oxidase did not simply consume protons on the matrix side of the mitochondrion; it actively pumped protons. How does one pump a proton from one side of a membrane to the other?

At a minimum, there must be recognition of the proton on side one of the membrane; there must be sequestration of the proton in the protein so that it no longer exchanges rapidly with the bulk solution; there must be recognition of the proton on side two of the membrane. Built into the recognition steps on the two sides of the membrane is the inherent idea that recognition uses protonatable residues that are probably amino acids. Also built into the recognition steps is the idea that if protonic activity in the bulk solution is greater than the pK of the recognizing group, substantial proton binding will occur; this must be the case on the matrix side of the mitochondrion during proton pumping. If the protonic activity of the bulk solution is less than the pK of the recognizing group, the recognition residue will surrender the proton to the bulk; this must be the case on the cytosolic face of the mitochondrion. Tentative residues that might function as gatekeepers for the oxidase at the two sides of the membrane have been proposed; some have been shown to function in the energy-transducing process (Mitchell et al., 1996; Hosler et al., 1996; Qian et al., 1997; Verkhovskaya et al., 1997; Konstantinov et al., 1997). Certainly, one of the crucial steps is the sequestration of the proton in the protein. This must involve, even if no other steps do, the energy available from electron transfer.

To return to the question of how one pumps a proton: part of the final solution must involve a mechanism for keeping the proton out of contact with the bulk solution on both sides of the membrane. As it is moved into the protein, does it migrate as a hydrated or a dehydrated species. It clearly does not enter a large void, because there are no large voids present in the crystal structure. Electron transfer could cause

TABLE 4 The inhibition of vesicular and soluble cytochrome *c* oxidase by 5 mM formamide

Sample	Activity, no formamide (ΔV/9 s)*	Activity, plus formamide (ΔV/9 s)	Normalized ratio of activities: inhibited soluble to inhibited vesicular oxidase#
Soluble oxidase 1 nM, control for no uncoupler	−0.0375	−0.0172	
Vesicular oxidase 1 nM, no uncouplers	−0.0151	−0.0091	76%
Soluble oxidase 0.25 nM control for uncouplers	−0.0125	−0.0053	
Vesicular oxidase + FCCP and valinomycin§	−0.0218	−0.0152	61%

*ΔV/9 s represents the change in voltage from the PM over the course of 9 s in a 1200-s assay.

#The normalized ratio of activities was computed as follows: ((Activity of soluble oxidase with formamide)/(Activity of soluble oxidase without formamide))/((Activity of vesicular oxidase with formamide)/(activity of vesicular oxidase without formamide)).

The activities were calculated when both soluble and vesicular oxidase samples had turned over the same number of times. The activities were calculated at four closely spaced times on the decay curve and averaged. A total of 1024 time points were taken for each curve. Fifteen time points were averaged at each of the four times.

§FCCP (Fluka) was used at a final concentration of 1 μM; valinomycin (Fluka) was used at a final concentration of 3 μM.

small voids to coalesce, thereby resulting in the construction of a functional pore. The physical bulk from amino acids, heme, copper, lipid, fatty acids, or water is not perfectly packed. It could be rearranged to form the needed space. There is at least circumstantial evidence that water must move for electron transfer to occur. The hydrostatic and osmotic stress experiments alluded to earlier both suggest that water must move for protons to move.

Formamide is a small compound about twice the size of water. Formamide has no effect on the Soret, visible, or UV spectra of the oxidized or reduced oxidase. During turnover it leads to a block in electron transfer between cyt *a* and the CuB/cyt a_3 center. Formamide shows a slow onset of inhibition that is independent of the concentration of cytochrome *c* oxidase but strictly dependent on the concentration or mole fraction of formamide and turnover of the oxidase.

On the assumption that formamide inhibits by competing with water, it appears as though one water is required for oxidase activity for each electron transferred in the oxidase. If formamide inhibits the cytochrome *c* oxidase by being mistaken for water and if the water in question is part of the proton-pumping channel, one predicts that coupled oxidase vesicles would show more activity in the presence of formamide than does soluble oxidase; this is the case. Protons are pumped from inside the vesicles to the outside. Treatment of vesicular oxidase with formamide leads to inhibition of activity, but the inhibition is less than that found with the soluble protein. Relative to the soluble protein, the vesicular oxidase, in the inhibited condition, is 1.4 times more active when the vesicles are in the coupled state and 2.2 times more active when in the uncoupled state. I have not yet taken any data that would negate the hypothesis that formamide inhibits the cytochrome *c* oxidase by entering the proton channel by accident. I speculate that while the proton channel is open to the inside of the vesicle, water or formamide enters along with the proton. The vesicle side closes and the proton is normally delivered to the external solution. Formamide is a little too large to get out easily. The result is that the channel stays blocked until such time as the formamide exits. If this scenario bears any relation to reality, it would mean that the proton channel had at least one gate, that for entry, that was somewhat larger than the formamide; a second gate, for exit, would a bit smaller than the first. A water/proton channel through the oxidase is not a novel idea. The x-ray structures of the bacterial photoreaction center (Ermler et al., 1994) and the cytochrome b_6f (Martinez et al., 1996) have both been shown to have water channels that either completely or partially traverse the membrane. These water channels are not identical and are not necessarily the same as that proposed for the cytochrome *c* oxidase; there is no reason to think that formamide will be an effective inhibitor of either. In the case of the photoreaction center, mutations that disrupt the water channel disrupt proton pumping (Baciou and Michel, 1995). It appears as though formamide, high hydrostatic pressure, and high osmotic pressure might do the same thing to the oxidase.

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