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Paradoxical Effects of Methylmercury on the Kinetics of Cytochrome *c* Oxidase†

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ABSTRACT: A stoichiometric amount of methylmercuric chloride substantially inhibits cytochrome *c* oxidase function under steady-state turnover conditions, where the enzyme is using its substrates, cytochrome *c* and oxygen, rapidly and continuously. Under these conditions, a reduction in activity of approximately 40% is observed. This is in accord with the results of Mann and Auer [Mann, A. J., & Auer, H. E. (1980) *J. Biol. Chem. 255*, 454–458], who used mercuric chloride and ethylmercuric chloride. Paradoxically, we found that addition of methylmercuric chloride can increase the activity of cytochrome *c* oxidase during its initial substrate utilization. This rate enhancement, measured under conditions where the enzyme cycles only a few times, is maximal for the resting state of the enzyme. "Pulsed" cytochrome *c* oxidase (i.e., enzyme that has been recently reduced and reoxidized) is considerably activated with respect to the resting enzyme, showing faster turnover rates (Antonini, 1977; Brunori et al., 1979). No significant rate enhancement upon treatment with methylmercuric chloride is seen in initial substrate utilization if the enzyme is pulsed immediately before the assay. The apparently contradictory effects of methylmercuric chloride on the resting and pulsed states of the oxidase under low turnover conditions may be reconciled by a model in which mercurial binding greatly stabilizes the enzyme in a state resembling that of the pulsed enzyme. A decrease in conformational flexibility may be the basis of the mercurial-induced diminution in activity of the enzyme during steady-state turnover conditions.

Many biological systems, e.g., enzymes, respiratory proteins, etc., have been reported to bind heavy metals. Binding of such metal atoms has also been shown to interfere with both the catalytic and regulatory functions of such systems. It is this binding that is presumably at the root of heavy-metal toxicity.

Cytochrome *c* oxidase, the terminal electron acceptor of the mitochondrial respiratory chain, plays a major role in cellular respiration. Organomercurials have no reported effects on the electron paramagnetic resonance (EPR) spectra of the enzyme (Beinert & Palmer, 1965). Mann & Auer (1980) have shown that the activity of cytochrome *c* oxidase drops to approximately 60% of its original value on binding a single atom of mercury per heme *a*, i.e., two Hg atoms per unit containing the full complement of four metal centers (two heme ions and two copper atoms). The activity of cytochrome *c* oxidase was reported to decrease sharply from the level of the control by addition of betwen 0 and 1 equiv of ethylmercuric chloride per heme *a*. Subsequent additions of ethylmercuric chloride up to 6 equiv per heme *a* had no further effect on the activity of the cytochrome *c* oxidase. The ethylmercury-modified enzyme maintains a relatively constant level of activity for at least several hours and, hence, is a stable modification (Mann & Auer, 1980). Mercury was shown to bind tightly to the enzyme and could not be removed by chromatographic techniques but only by thiol-exchange procedures. Because of its very high affinity for mercury, a sulfur-containing group almost certainly provides this mercury binding site (Mann & Auer, 1980; Darley-Usmar et al., 1981).

Sulfur atoms have been suggested to act as ligands for the copper atoms of cytochrome oxidase. For example, Blumberg & Peisach (1979) suggest that a copper atom liganded to sulfur is responsible for the EPR signal of the Cu center. Similarly, the amino acid sequence of subunit II shows homology with copper proteins (Steffens & Buse, 1979), particularly azurin and plastocyanin, again indicating that sulfur may act as a ligand to at least one of the copper atoms in this enzyme. In addition, Powers et al. (1981), on the basis of of EXAFS[1] data, proposed that in some states of the oxidized enzyme, sulfur may act as a bridging ligand between a copper atom (Cu_B) and the iron atom of cytochrome a_3, the oxygen binding site.

In order to explore further the mechanism of inhibition of cytochrome *c* oxidase by mercury, we have examined the effects of methylmercuric chloride (MeHgCl) on the activity

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; MeHgCl, methylmercuric chloride; EXAFS, extended X-ray absorbance fine structure.

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of the enzyme under a variety of turnover regimes. In particular, we have employed stopped-flow spectrophotometry to study the rapid oxidation of ferrocytochrome *c* in few-turnover experiments. Under these conditions, it is also possible to examine the effects of MeHgCl on different forms of the enzyme. It has been established that cytochrome *c* oxidase can exist in at least two forms (resting and pulsed), which differ in their ligand-binding and functional properties (Antonini et al., 1977) and their optical and EPR spectra (Brudvig et al., 1981; Armstrong et al., 1983), with the pulsed form being approximately 3–5-fold more active than the resting form. Interconversion between these forms has been suggested to account for activation of the enzyme during turnover and possibly to play a regulatory role in the control of enzyme activity (Colosimo et al., 1981). The effects of MeHgCl binding on the catalytic properties of both resting and pulsed cytochrome *c* oxidase have been examined.

Our results show that binding MeHgCl may have a number of different effects, depending upon the state of the enzyme and also upon whether the enzyme is turning over only a few times or has entered into true steady-state catalysis. A model is proposed that incorporates the known interconversions of the enzyme between states and that accounts for the effects of MeHgCl in terms of preferential stabilization of enzymic forms. The molecular basis of this model is discussed.

Materials and Methods

Submitochondrial, Keilin–Hartree particles were prepared from beef heart following the procedure of Yonetani (1961), except the initial grinding and washing stages to remove myoglobin were eliminated and the heart muscle was instead cut into small cubes. The cubes were homogenized in a blender with buffer, and the rest of the procedure was followed as reported. No spectrally detectable myoglobin contamination was found when this method was used.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was purified from the isolated submitochondrial particles. The procedure used was essentially that of Yonetani (1961), but the overnight precipitation step was limited to 6 h, and 1 mM EDTA was added to the buffers throughout. Care was taken that the entire procedure, from fresh heart to frozen, purified cytochrome *c* oxidase, take less than 24 h.

The quality of each enzyme preparation was determined by spectral criteria (Gibson et al., 1965; Kuboyama et al., 1972). Only enzyme preparations with 444- to 424-nm absorbance ratios of 2.25 or greater for the dithionite-reduced enzyme were used (Gibson et al., 1965). Enzyme preparations were tested in 0.1 M sodium phosphate with 1% Tween 80, pH 7.4.

Cytochrome *c* oxidase concentrations were determined in terms of total heme (heme a) by using a millimolar extinction coefficient of 21 for dithionite-reduced cytochrome *c* oxidase at 605 nm (Yonetani, 1960) and are expressed throughout in terms of functional units containing two hemes. Cytochrome *c* concentrations were determined spectrophotometrically in the presence of dithionite by using an E = 27.6 mM at 550 nm (Schejter et al., 1963).

The kinetics of cytochrome *c* oxidation were determined by stopped-flow spectrophotometry in a Gibson-Durrum stopped-flow spectrophotometer with a 1.46-cm observation chamber. Reduced cytochrome *c* was prepared by treating cytochrome *c* with 1 mM sodium ascorbate and, after reduction, removing the excess ascorbate by passage down a column (5 cm by 1 cm) of Sephadex G-25. The eluted cytochrome *c* was collected in a sealed septum bottle and then made anaerobic by 10 2-min cycles of evacuation and gas replacement with argon. Pulsed cytochrome *c* oxidase was prepared by adding 5 mM sodium ascorbate to an anaerobic solution of cytochrome *c* oxidase and cytochrome *c* and allowing time for reduction. Upon rapid mixing in the stopped flow with oxygen containing buffer, the cytochrome *c* oxidase is quickly oxidized to the pulsed form and can then oxidize the reduced cytochrome *c* by catalytic turnover.

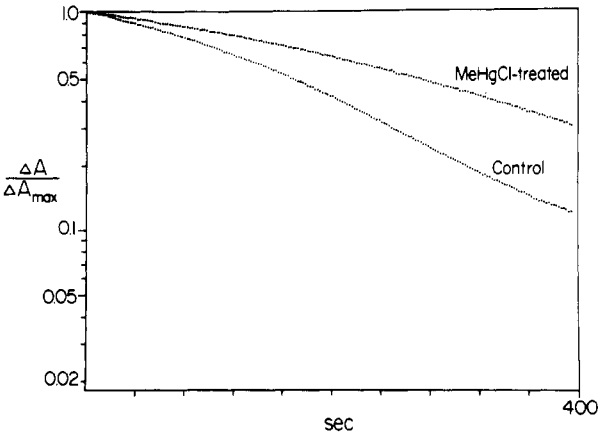


FIGURE 1: Effect of 1 molar equiv of MeHgCl on cytochrome *c* oxidation by cytochrome *c* oxidase under high-turnover conditions. Oxidized cytochrome *c* oxidase was mixed with anaerobic reduced cytochrome *c* at 20 °C. Concentrations after mixing: cytochrome *c* oxidase, 0.025 μM; cytochrome *c*, 50 μM; oxygen, 140 μM. Cytochrome *c* oxidation was observed at 550 nm. The buffer contained 0.1 M sodium phosphate–1% Tween 80, pH 7.5.

Polarographic assays of cytochrome *c* oxidase activity were performed in a water-jacketed polarographic cell (Rank Bros. Ltd.) at 20 °C. The assay medium contained 10 mM sodium ascorbate, 25 μM cytochrome *c*, 4 mM TMPD, and approximately 0.25 μM cytochrome *c* oxidase in the buffer described above.

Methylmercuric chloride (K & K Laboratories) was dissolved in ethanol to make a 0.1 M solution and added to the protein solutions with vigorous mixing. Unless otherwise stated, incubation was performed at a 1 to 5 concentration ratio of enzyme to MeHgCl. Cytochrome *c* (type III) was purchased from Sigma and used without further purification. All other reagents were reagent grade or better. Data collection and analysis were facilitated by use of a DASAR (American Instrument Co.) analogue/digital converter and a PDP 11/34 minicomputer (Digital Equipment Co.).

Results

It has been previously reported (Mann & Auer, 1980) that purified beef heart cytochrome *c* oxidase could be inactivated to the extent of 35–50% by the nonpolar mercurial reagents mercuric chloride and ethylmercuric chloride. We find that this inactivation also occurs with methylmercuric chloride (MeHgCl). A solution of cytochrome *c* oxidase was incubated with 2 molar equiv of MeHgCl. The activity of cytochrome *c* oxidase in electron transfer from cytochrome *c* to molecular oxygen was determined polarographically as described under Materials and Methods. The addition of 1 equiv of MeHgCl per heme *a* results in a 42% inhibition relative to the control.

Cytochrome *c* oxidase can also be shown to be inhibited by addition of MeHgCl when its function is monitored by stopped-flow spectrophotometry, following the absorption changes associated with cytochrome *c* oxidation. This is illustrated in Figure 1, where a large excess of cytochrome *c* relative to cytochrome *c* oxidase was present, allowing the determination of reaction rate under multiple-turnover conditions that approximate steady-state catalysis. In the specific

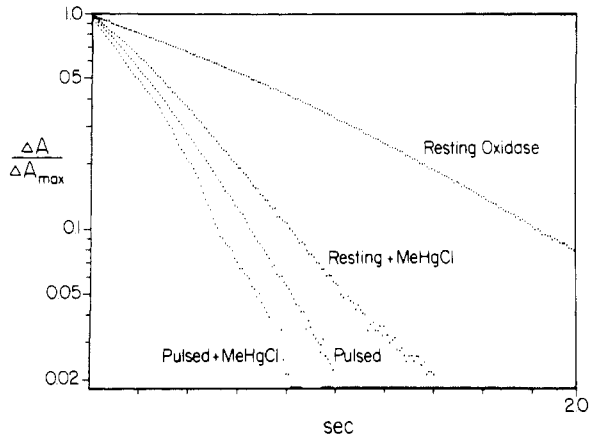


FIGURE 2: Effect of 1 molar equiv of MeHgCl on cytochrome *c* oxidation by resting and pulsed forms of cytochrome *c* oxidase under few-turnover conditions. Conditions are as in Figure 1, except cytochrome *c* oxidase was at 3 μM and cytochrome *c* at 15 μM. Pulsed cytochrome *c* oxidase was prepared as described in the text.

instance of Figure 1, the cytochrome *c* concentration was 50 μM, and the cytochrome *c* oxidase concentration was 0.025 μM after mixing. Thus, the reaction shown in Figure 1 represents a case where cytochrome *c* oxidase molecules must, on average, turnover 500 times and reduce 500 molecules of oxygen while oxidizing the cytochrome *c*.

Quite different results are obtained in response to MeHgCl addition when cytochrome *c* oxidation is observed under conditions where cytochrome *c* oxidase catalyzes the oxidation of only a few molecules of cytochrome *c*. The effect on the initial rate of enzyme turnover depends then on the immediate history of the enzyme. Consider first the behavior of oxidized cytochrome *c* oxidase in its resting configuration, the condition of the oxidized enzyme as isolated. Figure 2 shows that the initial rate of cytochrome *c* oxidation by resting cytochrome *c* oxidase is appreciably enhanced by prior incubation of the enzyme with MeHgCl. The same extent of activation by addition of MeHgCl was observed when the incubation period was varied from several minutes to several hours. In Figure 2, each cytochrome *c* oxidase molecule could, on average, reduce only 1.25 molecules of oxygen while oxidizing cytochrome *c*, because the cytochrome *c* and oxidase were at concentrations of 15 and 3 μM, respectively. The extent of activation by MeHgCl varied between 2- and 3-fold in different experiments under these conditions.

It is known that after cytochrome *c* oxidase has been pulsed (i.e., reduced and reoxidized immediately prior to an experiment), it is able to catalyze cytochrome *c* oxidation much more rapidly than is the enzyme as isolated (Antonini, 1977; Antonini & Brunori, 1978; Brunori et al., 1979). Under the particular conditions utilized in the present study, the pulsed enzyme shows a rate enhancement of a factor of 3 relative to the resting enzyme. In these experiments, the preparation of pulsed cytochrome *c* oxidase followed the procedure of Antonini & Brunori (1978) in which reduced cytochrome *c* oxidase is mixed with oxygen-containing buffer in the stopped-flow spectrophotometer (see Materials and Methods). The cytochrome *c* oxidase is rapidly oxidized to its pulsed state within the dead time of the instrument. The reduced cytochrome *c* may be either in the oxygen-containing buffer or with the reduced cytochrome *c* oxidase. In either case, following mixing, the reaction chamber contains recently oxidized (pulsed) enzyme in the presence of reduced cytochrome *c* and oxygen.

Resting and pulsed preparations respond differently to addition of MeHgCl. Figure 2 shows that treating pulsed cytochrome *c* oxidase with MeHgCl has only a modest effect on its initial rate of substrate utilization. This result was observed regardless of whether the MeHgCl was added to the cytochrome *c* oxidase before or after reduction. Thus, while preparations of resting cytochrome *c* oxidase show a 2–3-fold rate enhancement upon MeHgCl treatment, preparations of pulsed cytochrome *c* oxidase, already showing a 3-fold increase in rate over resting preparations, show no pronounced enhancement of reactivity in response to MeHgCl treatment.

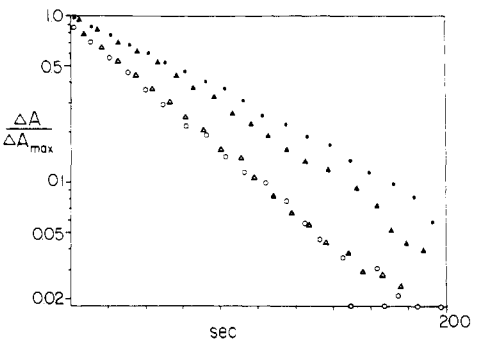


FIGURE 3: Effect of 100 μM MeHgCl on cytochrome *c* oxidation by resting and pulsed forms of cytochrome *c* oxidase in Keilin–Hartree particles. Concentrations after mixing: cytochrome *c* oxidase, 1.25 μM; cytochrome *c*, 5 μM; ascorbate, 5 mM, oxygen, 140 μM. The buffer contained 0.1 M $NaPO_4$–14 mM NaCl, pH 6.5. Cytochrome *c* oxidation was observed at 550 nm. (●) Resting control; (▲) resting with MeHgCl; (○) pulsed particles; (△) pulsed particles with MeHgCl.

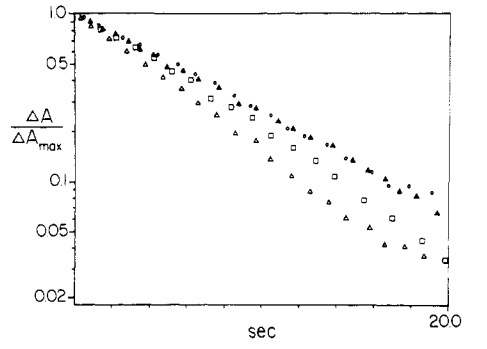


FIGURE 4: Effects of increasing concentrations of MeHgCl on cytochrome *c* oxidation by cytochrome *c* oxidase in Keilin–Hartree particles. Conditions are as in Figure 3, except with MeHgCl concentrations of (○) 0, (▲) 15, (□) 100, and (△) 1000 μM.

The addition of MeHgCl to cytochrome *c* prior to mixing with cytochrome *c* oxidase had no effect on cytochrome *c* oxidase catalyzed cytochrome *c* oxidation by either pulsed or resting cytochrome *c* oxidase. Thus, the rate differences are not linked to interactions of MeHgCl with cytochrome *c*. In addition, because resting cytochrome *c* oxidase, when mixed in the stopped flow with cytochrome *c* solutions containing MeHgCl, shows no rate enhancement under the minimum turnover conditions, we conclude that the interaction of MeHgCl with the resting enzyme that brings about rate enhancement is slow relative to the duration (several seconds) of the experiment.

The effects of MeHgCl shown above were observed with solutions of purified detergent-solubilized cytochrome *c* oxidase. Further experiments were performed to determine the effects of MeHgCl on the enzyme in its native mitochondrial membrane. It has been previously shown that cytochrome *c* oxidase in Keilin–Hartree particles can also be converted to the pulsed state (Bonaventura et al., 1978). Accordingly, parallel experiments to those performed with detergent-solubilized cytochrome *c* oxidase were performed with MeHgCl-treated Keilin–Hartree particles. As shown in

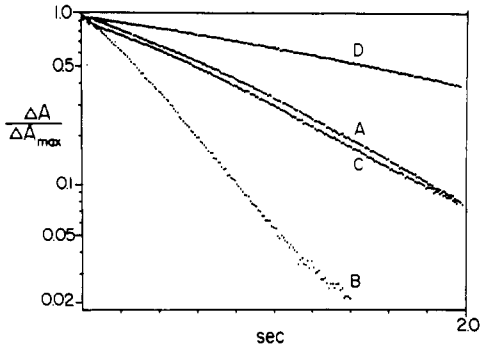


FIGURE 5: Effects of increasing MeHgCl concentrations on cytochrome *c* oxidation by detergent-solubilized resting cytochrome *c* oxidase. Enzyme concentrations after mixing: cytochrome *c* oxidase, 3 μM; cytochrome *c*, 3 μM. The cytochrome *c* oxidase was treated before mixing with MeHgCl concentrations of (A) 0, (B) 30, (C) 300, and (D) 3000 μM. Other conditions are as in Figure 1.

Figures 3 and 4, when Keilin–Hartree particles were allowed to react with cytochrome *c* under minimum turnover conditions, the rate of cytochrome *c* oxidation by the resting enzyme was enhanced by prior incubation of the particles with MeHgCl. When the particles were pulsed, the addition of MeHgCl had no effect on the rate of cytochrome *c* oxidation. It is apparent that for both the detergent-solubilized enzyme and the Keilin–Hartree particles the degree of rate enhancement brought about by MeHgCl is very similar to the degree of rate enhancement brought about by the transition to the pulsed condition.

It should be noted that in the experiments with Keilin–Hartree particles, the range of concentrations of MeHgCl where the maximum effect was observed was much higher than the concentrations used with the detergent-solubilized isolated enzyme. The higher concentrations used with the Keilin–Hartree particles (up to 1 mM) were introduced under the assumption that Keilin–Hartree particles have a significant number of sulfhydryl groups available for reaction with MeHgCl that are not involved in the cytochrome *c* oxidase catalyzed oxidation of cytochrome *c*. As shown in Figure 4, this assumption was borne out in our experiments, where even the highest concentration of MeHgCl we tested did not appear to saturate the activating effect of this compound.

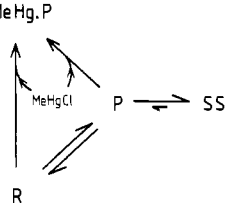
Comparable titrations of detergent-solubilized cytochrome *c* oxidase with MeHgCl showed that the enhancement of rate seen with the resting enzyme could be abolished if the mercury concentration was made sufficiently high. Figure 5 documents this result. At a cytochrome *c* oxidase concentration of 6 μM, treatment with 30 μM MeHgCl led to a 2–3-fold rate enhancement. At the same cytochrome *c* oxidase concentration, 300 μM MeHgCl (a 25-fold excess) had very little apparent effect on the enzyme activity, while 3000 μM MeHgCl led to a substantial inhibition of the enzyme, reducing its rate of cytochrome *c* oxidation to about half that of the untreated enzyme and to less than a fifth of the rate shown by the enzyme treated with a small excess of MeHgCl.

Discussion

The effects of a stoichiometric amount of MeHgCl on cytochrome *c* oxidase can be summarized as follows: In few-turnover experiments MeHgCl causes an activation of the resting enzyme but has little or no effect on the pulsed enzyme, and under steady-state turnover conditions, it causes a substantial inhibition of the enzyme. The enhancement of the initial rate of substrate utilization by resting cytochrome *c* oxidase is seen when the enzyme is either solubilized by detergent or in Keilin–Hartree particles. In either milieu, the rate shown by the pulsed enzyme is apparently unaffected by MeHgCl.

We may regard the cytochrome *c* oxidase catalyzed electron transfer from cytochrome *c* to oxygen as proceeding in three stages. The first stage is the binding of cytochrome *c* and electron transfer to cytochrome *a* and the associated copper (Cu_A). This is followed by an internal electron transfer from cytochrome *a* and Cu_A to cytochrome a_3 and its associated copper, Cu_B. Upon reduction of cytochrome a_3 and Cu_B, the final stage is the binding of oxygen and the transfer of two electrons from cytochrome a_3 and Cu_B to oxygen. Two more electrons are then transferred in order to complete the reduction of oxgen to water. The internal electron transfer from cytochrome *a* and Cu_A to cytochrome a_3 and Cu_B is the rate-limiting step, being several orders of magnitude slower than either the reduction of cytochrome *a* by cytochrome *c* or the reduction of oxygen by cytochrome a_3, when the reactants are near physiological concentrations (Brunori et al., 1981). Previously, one of us (H.A.) has shown that organic mercurials reduce the V_{max} of cytochrome *c* oxidation by cytochrome *c* oxidase. The K_m for the reaction is unchanged, indicating that organic materials do not affect the affinity of cytochrome *c* oxidase for cytochrome *c*. The aggregation state of cytochrome *c* oxidase also is unaffected by organic mercurials, as is the ionic strength dependence of the enzyme activity (Mann & Auer, 1980). The decrease in V_{max} upon treatment with organic mercurials, taken with their lack of effect on other parameters, suggests that organic mercurials exert their influence upon the rate-limiting step, the internal electron transfer. The rate of internal electron transfer has been shown to be enhanced in pulsed cytochrome *c* oxidase (Antonini et al., 1977). Under low turnover conditions, MeHgCl brings about a comparable degree of activation to that which occurs when resting cytochrome *c* oxidase is converted to the pulsed form but has little or no effect on the rate of cytochrome *c* oxidation by the pulsed enzyme. These two observations suggest that MeHgCl acts to limit the ability of cytochrome *c* oxidase to adopt different forms, effectively freezing cytochrome *c* oxidase in a pulsed-like form.

The following mechanism, which is no doubt a gross simplification, attempts to rationalize the apparently paradoxical effects of both activation and inhibition of MeHgCl on the basis of known interconversions between states of oxidase possessing differing catalytic properties:



In experiments where the enzyme is allowed to turnover only a few times, the steady-state species (SS) is sparsely populated, and the characteristic catalytic properties of the R or P forms (or combinations of these) are displayed. In many-turnover experiments, where the enzyme-catalyzed reactions approach a steady state, the major form of the enzyme is that termed SS. This form of the enzyme may be characterized by an approximately 40% greater activity than the pulsed enzyme. Such a form of the enzyme has been previously postulated by Brunori et al. (1979) on the basis of spectral observations.

Addition of MeHgCl to either the oxidized resting or reduced enzyme results in a species that is similar in activity to the pulsed form (P). In other words, MeHgCl acts to trap cytochrome *c* oxidase in a pulsedlike state, which is more active

than the resting yet less active than the steady-state form of the enzyme.

To propose a mechanism through which such stabilization may be achieved demands knowledge of the Hg-binding location within the enzyme. Darley-Usmar et al. (1981) have shown that virtually all bound ^{203}Hg resides on subunit II, when the native enzyme is exposed to $HgCl_2$ at a ratio of 1.5 $HgCl_2$ to 1 heme. It has also been demonstrated (Darley-Usmar, 1980), that MeHgCl binds preferentially to subunit II under the conditions used by Mann & Auer (1980). The mercury can be removed from this subunit by treatment with sulfhydryl compounds such as mercaptoethanol or 2,3-dimercaptopropanol (Mann & Auer, 1980; Darley-Usmar et al., 1981). It is known that subunit II contains two sulfhydryl groups, one of which is thought to be a ligand for one of the copper atoms. Although it is a matter of debate whether Cu_A or Cu_B is associated with subunit II, the lack of effect of mercury binding on the optical and EPR spectra of Cu_A suggests that it is Cu_B that is associated with this subunit. In this case, the way methylmercury stabilizes a pulsed-like form may be by binding to a specific sulfur atom in the vicinity of the oxygen binding site. Recent EXAFS studies of resting cytochrome *c* oxidase indicate that an endogenous sulfur ligand bridges the iron of cytochrome a_3 and its associated copper atom (Cu_B) (Powers et al., 1981). This is relevant to the mechanism described above. The available evidence suggests that the endogenous bridge is broken in the reduced and the pulsed forms of the enzyme (Chance et al., 1982). As MeHgCl is a relatively small, nonpolar compound with a high affinity for sulfur, it may be capable of entering the active site and disrupting the sulfur bridge, thus producing a pulsed-like form of the enzyme from the resting state. If the action of Hg is indeed to disrupt a sulfur bridge, then these findings suggest that subunit II provides the bridging ligand between Cu_B and the heme of cytochrome a_3.

Of course, there are alternative ways to stabilize the pulsed form to that discussed above. Any conformational change between the resting and pulsed forms that placed bound MeHg in a thermodynamically more favorable environment in the P state would tend to stabilize this form. Thus it may be premature to conclude from our data that subunit II is necessarily that associated with Cu_B. If, however, the Cu_B coordination shell is perturbed by binding of MeHg to subunit II, this should be apparent from the EXAFS spectrum of the mercury-bound enzyme.

In summary, during few-turnover experiments MeHgCl-treated resting enzyme exhibits an increased activity similar to that expected for the pulsed (P) enzyme. In many-turnover experiments, because this P-type configuration is stabilized, transition to the SS form is hindered (either due to kinetic reasons or because the transition to SS is no longer favored thermodynamically). The activation resulting from transition to the SS form is thus prevented, and the enzyme shows an apparent 40% inhibition when compared with untreated samples.

Registry No. Methylmercuric chloride, 115-09-3; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6.

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