The Inhibition of Mitochondrial Complex I (NADH:Ubiquinone Oxidoreductase) by Zn$^{2+}$

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NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria is a highly complicated, membrane-bound enzyme. It is central to energy transduction, an important source of cellular reactive oxygen species, and its dysfunction is implicated in neurodegenerative and muscular diseases and in aging. Here, we describe the effects of Zn$^{2+}$ on complex I to define whether complex I may contribute to mediating the pathological effects of zinc in states such as ischemia and to determine whether complex I may contribute to mediating the pathological effects of zinc in states such as ischemia and to determine how Zn$^{2+}$ can be used to probe the mechanism of complex I. Zn$^{2+}$ inhibits complex I more strongly than Mg$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, and Mn$^{2+}$ to Cu$^{2+}$ or Cd$^{2+}$. It does not inhibit NADH oxidation or intramolecular electron transfer, so it probably inhibits either proton transfer to bound quinone or proton translocation. Thus, zinc represents a new class of complex I inhibitor clearly distinct from the many ubiquinone site inhibitors. No evidence for increased superoxide production by zinc-inhibited complex I was detected. Zinc binding to complex I is mechanistically complicated. During catalysis, zinc binds slowly and progressively, but it binds rapidly and tightly to the resting state(s) of the enzyme. Reactivation of the inhibited enzyme upon the addition of EDTA is slow, and inhibition is only partially reversible. The IC$_{50}$ value for the Zn$^{2+}$ inhibition of complex I is high (10–50 µM, depending on the enzyme state); therefore, complex I is unlikely to be a major site for zinc inhibition of the electron transport chain. However, the slow response of complex I to a change in Zn$^{2+}$ concentration may enhance any physiological consequences.

Mitochondria play central roles in crucial cellular processes such as ATP production, the regulation of intracellular calcium levels, and apoptosis, and thus the disruption of normal mitochondrial function is central to neurodegenerative and muscular diseases and aging (1–3). High intracellular free zinc levels have been implicated in neuronal cell death, for example, following cerebral ischemia and mechanical trauma and in Alzheimer disease (4–6). The precise mechanism of zinc cytotoxicity is unknown, but zinc is known to inhibit cellular energy production, causing loss of the mitochondrial membrane potential and/or an increase in the production of reactive oxygen species (7–9). Zinc has been proposed to inhibit mitochondrial trafficking (10), the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (11), and the α-ketoglutarate dehydrogenase complex of the tricarboxylic acid cycle (12, 13). It is known to inhibit the electron transport chain in isolated mitochondria and mitochondrial membrane preparations (14, 15), and specific studies have demonstrated that the cytochrome bc$_1$ complex (16, 17) and cytochrome c oxidase (18, 19) are both inhibited by micromolar concentrations of zinc.

The effects of zinc on mitochondrial NADH:ubiquinone oxidoreductase (complex I) have not been described previously, although complex I is both central to energy transduction and an important source of cellular reactive oxygen species (20, 21). It is the first enzyme of the mitochondrial respiratory chain, catalyzing NADH oxidation in the mitochondrial matrix and ubiquinone reduction in the inner mitochondrial membrane, coupled to transmembrane proton translocation (22, 23). Complex I from bovine mitochondria is a highly complicated enzyme comprising 45 different subunits (24) and 9 redox cofactors (25, 26), a non-covalently bound FMN, and 8 iron-sulfur clusters. Recently, the structure of the hydrophilic domain of a simpler bacterial complex I confirmed that the clusters form a conduit for electrons between the sites of NADH oxidation and a proposed site for ubiquinone reduction at the interface with the membrane domain (26, 27) (Fig. 1), but the mechanisms of quinone reduction and proton translocation remain unknown. Similarly, questions remain concerning the mechanism of superoxide production. It is likely that superoxide is produced by the reduced flavin (28), but a second reactive site, perhaps a bound semiquinone, has been proposed to make a significant contribution to reactive oxygen species production under certain conditions (29).

The strategy developed here is to use highly pure and catalytically active isolated complex I (30) as a simple experimental system that allows precise measurements of the effects of zinc on both the NADH:ubiquinone oxidoreductase activity and on the production of superoxide (28). The aims of this work are thus 2-fold: first, to define the limits and possibilities for the role of complex I in zinc-mediated pathologies by determining the timescale and potency of zinc inhibition; and second, to define the molecular mechanism of zinc inhibition to further investigate the catalytic mechanism of complex I. In particular, zinc binding has proven valuable in localizing and elucidating the mechanisms of proton transfer in the cytochrome bc$_1$ complex (17, 31) and cytochrome c
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![Diagram of complex I inhibition by Zn$^{2+}$](image)

**FIGURE 1.** Representation of the reaction mechanism of complex I showing the four points at which Zn$^{2+}$ binding may inhibit catalysis, NADH binding or oxidation, intramolecular electron transport, quinone binding or reduction, or proton translocation.

oxidase (18, 19, 32–35), whereas the mechanism of proton transfer in complex I remains undetermined.

**EXPERIMENTAL PROCEDURES**

**Preparation of Complex I from Bovine Heart Mitochondria**—Complex I was prepared from bovine heart mitochondria as described previously (30), however, with minor modifications. EDTA was omitted from all buffers, and HEPES was used instead of Tris-HCl to minimize the chelation of divalent metal cations (M$^{2+}$).

**Catalytic Activity Assays**—The NADH:decylubiquinone oxidoreductase activity of complex I was determined in 20 mM HEPES-KOH (pH 7.5) and 0.5 mg ml$^{-1}$ asolectin at 32 °C, unless stated otherwise. 100 μM NADH (Sigma, from a 10 mM stock solution) and 200 μM decylubiquinone (Sigma, from a 35 mM stock solution) were used as substrates. The high decylubiquinone concentration helps to avoid limitations from substrate availability as the assays progress. Typically, 5 μg of complex I were used. The reaction was monitored via the NADH depletion at 340–380 nm ($ε = 4.81\,\text{mm}^{-1}\text{cm}^{-1}$), and initial rates are specific activities reported per milligram of complex I (determined using the FMN concentration). For complex I, 1 mg is equal to 1 nmol, as the molecular mass is ~1 MDa (24). When complex I was incubated at 32 °C prior to initiation of catalysis, it was incubated at its final concentration in the assay buffer with phospholipids but in the absence of substrates. Then, 200 μM decylubiquinone was added, the absorbance was allowed to stabilize, and 100 μM NADH was added to initiate turnover. When required, superoxide dismutase (SOD; Cu,Zn-SOD from bovine erythrocytes, Sigma) was added to 10 units ml$^{-1}$. The NADH:hexammineruthenium (HAR) oxidoreductase activity was determined in 20 mM HEPES-KOH (pH 7.5) using 1.6 μg of complex I, 1.4 mM HAR, and 140 μM NADH. The reaction was initiated by the addition of NADH and monitored by the decrease in NADH concentration (340 nm; $ε = 6.22\,\text{mm}^{-1}\text{cm}^{-1}$). H$_2$O$_2$ was quantified using the horseradish peroxidase (MP Biomedicals; 2 units ml$^{-1}$)-dependent oxidation of Amplex Red at a concentration of 10 μM (Invitrogen) to resorufin ($ε_{557–620} = 51.6 \pm 2.5\,\text{mm}^{-1}\text{cm}^{-1}$ at pH 7.5) (28).

**EPR Spectroscopy**—Spectra were recorded on a Bruker EMX X-band spectrometer using an ER 4119HS high sensitivity cavity maintained at low temperature by an ESR900 continuous flow liquid helium cryostat (Oxford Instruments); the sample temperature was measured by a calibrated Cernox resistor (Lake Shore Cryotronics, Inc.).

**RESULTS**

**The Effect of Divalent Cations on the NADH:Decylubiquinone Oxidoreductase Activity of Complex I**—Fig. 2 presents the effects of a range of divalent metal cations (M$^{2+}$) on the catalytic activity of complex I purified from bovine heart mitochondria. The activities reported are initial rate measurements, and they are all sensitive to inhibition by rotenone, confirming that ubiquinone reduction is occurring at the physiological (energy-transducing) binding site. The specific activity in the absence of M$^{2+}$, ~8 μmol e$^-\min^{-1}\text{mg}^{-1}$, is equal to that of complex I in the inner mitochondrial membrane (30). Clearly, the different cations inhibit to varying extents; M$^{2+}$ from Group 2 (Mg$^{2+}$, Ca$^{2+}$, and Ba$^{2+}$) exert little effect on the initial rate, whereas the inhibitory potency of M$^{2+}$ from the first row transition elements follows approximately the order across the row from Mn$^{2+}$ to Zn$^{2+}$. The trend probably reflects increased electrostatic interactions modulated by ligand field effects. Clearly, Zn$^{2+}$ is the most potent inhibitor, although Cd$^{2+}$, below Zn$^{2+}$ in Group 12, is also effective. A similar trend was reported previously for modulation of the catalytic activity of cytochrome $c$ oxidase by divalent cations (33). The concentration dependence of the Zn$^{2+}$ inhibition is described below. Finally, the Zn$^{2+}$ inhibition of complex I was confirmed in experiments using mitochondrial membrane preparations also. All results
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**Fig. 1.** The mechanism of energy transduction in complex I and identifies four points at which it may be inhibited by Zn\(^{2+}\): (i) the binding or oxidation of NADH, (ii) intramolecular electron transfer from the FMN to bound quinone, (iii) ubiquinone binding or reduction, and (iv) proton translocation. Note that all of the processes are tightly coupled, so that simple inhibition at any point should prevent NADH-decylubiquinone oxidoreductase catalysis completely; inhibiting proton transfer should prevent NADH oxidation just as effectively as inhibiting NADH binding.

First, HAR is an artificial electron acceptor that accepts electrons from complex I “upstream” of the physiological ubiquinone binding site, decoupling NADH oxidation from ubiquinone reduction and proton translocation. Fig. 3A shows that the NADH:HAR oxidoreductase activity of complex I is not compromised by Zn\(^{2+}\), even if the enzyme is preincubated with Zn\(^{2+}\) for 30 min before the assay is initiated. Therefore, Zn\(^{2+}\) does not inhibit the binding or oxidation of NADH.

Second, complex I was incubated overnight at 4 °C in the presence of 50 μM Zn\(^{2+}\). Then, it was reduced anaerobically using 5 mM NADH, frozen immediately, and evaluated by EPR spectroscopy. The spectra (not shown) demonstrate clearly that cluster N2, the final cluster in the chain between the FMN and the ubiquinone binding site, is fully reduced. They are essentially identical to spectra from a control sample and also to previously described spectra from bovine complex I (25), showing that none of the other EPR-detectable clusters are affected either. Thus, it is unlikely that Zn\(^{2+}\) inhibits electron transfer from the FMN to cluster N2 or that a Zn\(^{2+}\) derivative of one of the iron-sulfur clusters has been formed (36). However, it is important to note that a decrease in the rate of electron transfer, which if severe enough could impact on the rate of catalysis, may remain undetected by this “end point” experiment.

Third, to investigate whether Zn\(^{2+}\) binding competes with or affects ubiquinone binding, the NADH:decylubiquinone oxidoreductase activity was determined over a range of decylubiquinone concentrations in the presence of Zn\(^{2+}\) (Fig. 3B). The reduction of decylubiquinone is rotenone-sensitive (≈97%) (30, 37) and coupled to proton translocation (37); therefore, these assays address ubiquinone binding at the physiologically relevant site. Increasing the decylubiquinone concentration does not relieve Zn\(^{2+}\) inhibition, and although Zn\(^{2+}\) inhibition is not readily reversible (see below), Zn\(^{2+}\) affects the apparent \(k_{cat}\) and not the apparent \(K_m\) for decylubiquinone reduction. Therefore, Zn\(^{2+}\) binding does not interfere with ubiquinone binding to complex I.

Fourth, Fig. 3C shows that inhibition by Zn\(^{2+}\) is strongly pH-dependent. Although a single pK value cannot be derived from the data, Fig. 3C suggests that Zn\(^{2+}\) competes with H\(^{+}\) for one or more protonatable ligands. Zn\(^{2+}\) binding may disrupt many different components of the mechanism, including proton transfer to bound quinone, gated proton transfer across a hydrophobic barrier, proton transfer through a channel, or a conformational change. Unfortunately, no reproducible method for reconstituting isolated bovine complex I into proteoliposomes exists, precluding direct investigation of the effects of the proton motive force at present.

**In summary,** Zn\(^{2+}\) does not inhibit complex I by inhibiting NADH oxidation, by impeding electron transfer along the
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**FIGURE 4.** Inhibition of the NADH:decylubiquinone oxidoreductase activity of complex I by Zn\textsuperscript{2+} increases progressively during turnover. The assay medium comprised 20 mM HEPES, pH 7.5, 100 \( \mu \)M NADH, 200 \( \mu \)M decylubiquinone, and 0.5 mg ml\textsuperscript{-1} asolectin, and the reaction was initiated by 5 \( \mu \)g of bovine complex I. The NADH concentration was monitored at 340–380 nm (\( \varepsilon = 4.8 \text{ mm}^{-1} \text{ cm}^{-1} \)). Abs., absorbance.

The progressive increase in Zn\textsuperscript{2+} inhibition of Complex I during turnover—Fig. 4 shows extended assay traces recorded following the initiation of the NADH:decylubiquinone oxidoreductase reaction by the addition of complex I in the presence of Zn\textsuperscript{2+}. The traces are significantly curved showing that inhibition increases gradually during turnover. The most obvious explanation is that Zn\textsuperscript{2+} binds to complex I slowly. However, Fig. 5A compares initial rates of catalysis as a function of [Zn\textsuperscript{2+}] when the reaction is initiated by the addition of complex I and when complex I is incubated at 30 °C for 30 min in the presence of Zn\textsuperscript{2+} and the reaction is initiated by the addition of the substrates. Although preincubation causes the IC\textsubscript{50} value to decrease from \( \sim 55 \) to \( \sim 10 \ \mu \text{M} \) Zn\textsuperscript{2+}, separate experiments exclude the simple interpretation that Zn\textsuperscript{2+} binding is slow; the same level of inhibition is observed when complex I is incubated for 30 min in the absence of Zn\textsuperscript{2+} and then Zn\textsuperscript{2+} is added immediately before turnover is initiated (Fig. 5B). These observations suggest that, during incubation at 30 °C, the enzyme slowly converts to a form that is able to coordinate Zn\textsuperscript{2+} more rapidly or more tightly.

However, a slow conversion between two states over 30 min cannot explain the progressive inhibition observed during catalysis. Short preincubations with Zn\textsuperscript{2+} did not achieve the same degree of inhibition as an equivalent time of exposure to Zn\textsuperscript{2+} during turnover. Furthermore, the reactivation of Zn\textsuperscript{2+}-bound complex I upon the addition of EDTA is slow, and full reactivation, once the enzyme has catalyzed substrate conversion in the presence of Zn\textsuperscript{2+}, cannot be achieved on a viable experimental timescale. As expected, the effects of Zn\textsuperscript{2+} binding are reversed more effectively, following preincubation of complex I and Zn\textsuperscript{2+}, when EDTA is added 15 min before initiation (EDTA alone does not affect the catalytic activity). Importantly, the limited reversibility is not because of enzyme precipitation (Zn\textsuperscript{2+} binding does not affect the NADH:HAR oxidoreductase activity) (Fig. 3A) or delipidation (Zn\textsuperscript{2+} inhibition is not alleviated by additional phospholipids). We propose two possible explanations explored further below, which are consistent with our observations. (i) Following initial weak association of Zn\textsuperscript{2+} and complex I, conversion to a “tight binding” state occurs slowly (zinc-induced isomerization); during turnover the conversion is promoted by a catalytic step (38–40). (ii) Catalysis by Zn\textsuperscript{2+}-bound complex I is a form of “suicide inactivation.” Possibilities include Zn\textsuperscript{2+} being “pumped” irreversibly into a proton channel and the increased production of reactive oxygen species, which damage the enzyme and initiate a vicious cycle.

The Relationship between Cation Binding and Enzyme State—Although the effects of Zn\textsuperscript{2+} on isolated complex I have not been described, the interactions of complex I with other divalent cations, particularly Ca\textsuperscript{2+}, have been studied previously (41). Complex I has been proposed to exist in two distinct forms, “active” and “deactive” (42–44). The enzyme deactivates slowly in the absence of turnover and is reactivated slowly when catalysis is initiated by the addition of substrates. The active
enzyme was proposed to be insensitive to divalent cations (Ca\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), La\(^{3+}\), Mn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), and Ba\(^{2+}\)), whereas they bind to and trap the deactive state.

Fig. 6 illustrates the fact that the effects of Zn\(^{2+}\) and Ca\(^{2+}\) on complex I are qualitatively very similar but that their relative binding affinities are very different. In Fig. 6, a comparison of sets of assay traces recorded following a 30-min incubation indicates that the complex is ~200-fold more sensitive to inhibition by Zn\(^{2+}\) than Ca\(^{2+}\), consistent with the high (mM) binding constant previously reported for Ca\(^{2+}\) binding to the deactive state (41) and with the lack of observable effect from Ca\(^{2+}\) on the active enzyme. Thus, models in which M\(^{2+}\) bind exclusively to the deactive state reflect the heightened susceptibility of this state but are not consistent with the direct effects of Zn\(^{2+}\) during turnover, and they do not account for the progressive increase in inhibition observed. Although the clear similarity between the effects of Zn\(^{2+}\), as described above, and Ca\(^{2+}\), as described previously, suggests that a common mechanism may be applied to both cations, we refer here to the “resting” rather than to the deactive state to differentiate the interpretations of our study from those of previous studies.

The Effects of Zn\(^{2+}\) on Superoxide Production by Complex I—Zinc binding may affect the production of superoxide by complex I, but as proposed previously for Ca\(^{2+}\) (45), it may also exacerbate the damage that superoxide causes to the enzyme. First, no increase in H\(_2\)O\(_2\) production by complex I upon Zn\(^{2+}\) binding could be detected by the Amplex Red assay, either in the presence of NADH only or during NADH:ubiquinone oxidoreduction (data not shown). Therefore, Zn\(^{2+}\) binding does not increase the production of superoxide from the reduced flavin in complex I (28). Second, the addition of SOD had no effect on inhibition of the NADH:ubiquinone oxidoreductase activity by Zn\(^{2+}\), and identical results were obtained in aerobic and anaerobic (O\(_2\) ~2 parts/million) environments, suggesting that the progressive increase in inhibition observed here is not because of oxidative damage.

**DISCUSSION**

Is Complex I Inhibition Relevant to Zn\(^{2+}\)-mediated Pathologies?—Complex I is inhibited by micromolar concentrations of Zn\(^{2+}\); however, because inhibition is slow (on the experimental timescale), and complicated by changes in the enzyme that occur independently during preincubation, a thermodynamic binding constant cannot be determined. Instead, apparent IC\(_{50}\) values of 50 \(\mu\)M (no preincubation) and 10 \(\mu\)M (30-min preincubation) are reported (Fig. 5A); the IC\(_{50}\) value depends on the preincubation time and decreases as it is extended (Fig. 5B). Thus, it is clear that Zn\(^{2+}\) binding will not occur under normal conditions when free Zn\(^{2+}\) levels are tightly controlled and very low (if not zero) (46) and that a significant effect is only possible, if at all, when Zn\(^{2+}\) concentrations are elevated during pathological situations such as ischemia (4–6). Interestingly, the sensitivity of complex I to zinc probably increases under these conditions, when the enzyme is not fully catalytically active. Note that the existence of the deactive state remains to be demonstrated in vivo but that it has been reported in studies of anoxia using rat hearts (47) and in isolated mitochondria (44). Finally, comparison of the IC\(_{50}\) values determined here with the zinc binding constants determined for the cytochrome bc\(_{1}\) complex (0.1–3 \(\mu\)M (16, 17, 31)) and cytochrome c oxidase (1–10 \(\mu\)M (18, 33–35)) shows that the apparent affinity of complex I for zinc overlaps with the higher end of the range of values reported. The inhibition of complex I may gain additional physiological significance from its strong influence on the overall rate of ATP synthesis and from the slow recovery time of the inhibited enzyme.

The results presented here suggest that Zn\(^{2+}\) affects complex I by impairing its catalytic activity rather than by causing an increase in superoxide production. Previously, Sadek et al. (45) have shown that the NADH:ubiquinone-1 oxidoreductase activity of complex I in solubilized mitochondria is inhibited by Ca\(^{2+}\) (but to a maximum of 35% inhibition) in the presence of NADH and that the inhibition cannot be reversed by chelation (consistent with the lack of reversibility observed here). The observed loss in activity was prevented by SOD, and activity could be restored either by dithiothreitol or by turnover following Ca\(^{2+}\) chelation. It was suggested that Ca\(^{2+}\) binding alters the conformation of the enzyme, exposing a cysteine residue to oxidative modification. The apparent discrepancy with our results (which are not affected by the presence of SOD) is unlikely to arise from the different effects of Zn\(^{2+}\) or Ca\(^{2+}\) (Fig. 6). It is probably due to the involvement of other mitochondrial...
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Inhibitory mechanisms of Complex I by Zn

Zn inhibits complex I by inhibiting either quinone reduction (perhaps by inhibiting proton transfer to the quinone site) or proton translocation. Thus, as an inhibitor, Zn is clearly distinct from the myriad of complex I “Q-site” inhibitors. Similar modes of Zn inhibition have been observed in many other proton-translocating membrane proteins. Zn inhibits the bacterial photosynthetic reaction center by binding to amino acid residues that mediate proton transfer to Q (51, 52); it probably inhibits the cytochrome b6f complex by preventing proton egress from the Q site (17, 31), and it inhibits proton transfer in cytochrome c oxidase by binding at the entrance to one or more proton channels (18, 32, 33, 35). In the future, Zn binding may be applied to localize proton channels or other key structural catalytic features in complex I in a similar fashion. Other proton-transferring proteins inhibited by Zn++ include the proton-translocating transhydrogenase from Escherichia coli (53) and voltage-gated proton channels (54, 55).

The mechanism by which Zn binds to complex I is complicated. First, complex I exists in two different states, with the “high affinity, low activity” state being formed slowly in the absence of turnover. Interestingly, a number of distinct states have been reported for cytochrome c oxidase also (56–59). As with complex I, a “fast” form predominates during normal turnover, and resting or “slow” forms, generated in the absence of turnover, may be converted back to the fast form by reduction and reoxidation. In complex I, it is not clear whether the resting or deactive state is a single specific state, as proposed previously (43), or whether it comprises a distribution of states. Indeed, the high apparent activation energies determined for the “A to D” transition (for example, 270 kJ mol\(^{-1}\) for bovine complex I (42, 60)) and the unreasonably high pre-exponential factors that are implicit in these calculations (~10\(^{43}\) s\(^{-1}\)) for the bovine enzyme using \(t_{95} = 15\) min at 30°C (43)) do not support a simple two-state conversion and must, at least, reflect transition across a complex energy surface.

Second, inhibition increases progressively during catalysis. Increased superoxide production/oxidative damage has been ruled out as the cause in our experiments, but a different mechanism of suicide inactivation remains a possibility. For example, Zn may be progressively “taken up” into a proton channel, because catalysis drives proton (or Zn\(^{2+}\)) transfer in only one direction (61) or, as observed in cytochrome c oxidase (62), prevention of proton transfer to the complex I active site may promote side reactions that damage the active site and preclude further turnovers. However, it is perhaps more likely that the progressive inhibition results from Zn binding to a specific enzyme state, as observed for Zn++ and Cd++ inhibition of a number of ion channels. For example, Cd++ binds to the external mouth of the Shaker K+ channel pore with >10\(^4\)-fold higher affinity for the inactive state than for the open state (63); Zn++ inhibits Torpedo electric organ CIC-0 chloride channels by binding with high affinity to an inactivating state, with low affinity binding to the open state promoting its conversion to the inactive state (64); and Zn++ also inhibits the human muscle CIC-1 chloride channel by binding with high affinity to a closed state (65).

Consequently, we propose the following model for the inhibition of complex I by Zn++. In the absence of turnover, at 30°C, complex I relaxes to form a distribution of “resting states.” Relaxation may comprise the partial and reversible unfolding of a flexible active site region, supported by the structurally stable protein scaffold (66, 67). The resting states are conformationally mobile and so are accessible to either zinc or substrate binding. Subsequently, on the addition of NADH, they either reorganize around the bound cation, locking it into the site, or reform the higher energy catalytic states, returning to the catalytic cycle. Interestingly, a sulfhydryl group becomes accessible to derivatization by N-ethylmaleimide in deactive complex I and may form part of the nascent zinc binding site (68). Conversely, during turnover, the tightly controlled conformations of the catalytic intermediates are less susceptible to Zn++ binding, which is slowed considerably. Zn++ binds via a weak interaction with one or more active states followed by slow conversion to a tightly bound state, which may or may not equate to the Zn++-bound state formed from the resting states.

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**34808 JOURNAL OF BIOLOGICAL CHEMISTRY**

**VOLUME 281 • NUMBER 46 • NOVEMBER 17, 2006**
