Zinc Is a Potent Inhibitor of Thiol Oxidoreductase Activity and Stimulates Reactive Oxygen Species Production by Lipoamide Dehydrogenase*

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Submicromolar zinc inhibits α-ketoglutarate-dependent mitochondrial respiration. This was attributed to inhibition of the α-ketoglutarate dehydrogenase complex (Brown, A. M., Kristal, B. S., Effron, M. S., Shestopalov, A. I., Ullucci, P. A., Sheu, K.-F. R., Blass, J. P., and Cooper, A. J. L. (2000) J. Biol. Chem. 275, 13441–13447). Lipoamide dehydrogenase, a component of the α-ketoglutarate dehydrogenase complex and two other mitochondrial complexes, catalyzes the transfer of reducing equivalents from the bound dihydrolipoate of the neighboring dihydrolipoamide acyltransferase subunit to NAD⁺. This reversible reaction involves two reaction centers: a thiol pair, which accepts electrons from dihydrolipoate, and a non-covalently bound FAD moiety, which transfers electrons to NAD⁺. The lipoamide dehydrogenase reaction catalyzed by the purified pig heart enzyme is strongly inhibited by Zn²⁺ (Kᵢ –0.15 μM) in both directions. Steady-state kinetic studies revealed that Zn²⁺ competes with oxidized lipoamide for the two-electron-reduced enzyme. Interaction of Zn²⁺ with the two-electron-reduced enzyme was directly detected in anaerobic stopped-flow experiments. Lipoamide dehydrogenase also catalyzes NADH oxidation by oxygen, yielding hydrogen peroxide as the major product and superoxide radical as a minor product. Zn²⁺ accelerates the oxidase reaction up to 5-fold with an activation constant of 0.09 ± 0.02 μM. Activation is a consequence of Zn²⁺ binding to the reduced catalytic thiolis, which prevents delocalization of the reducing equivalents between catalytic disulfide and FAD. A kinetic scheme that satisfactorily describes the observed effects has been developed and applied to determine a number of enzyme kinetic parameters in the oxidase reaction. The distinct effects of Zn²⁺ on different LADH activities represent a novel example of a reversible switch in enzyme specificity that is modulated by metal ion binding. These results suggest that Zn²⁺ can interfere with mitochondrial antioxidant production and may also stimulate production of reactive oxygen species by a novel mechanism.

A number of reports suggest that mobilization of intracellular Zn²⁺ may play a role in cellular toxicity following ischemia-reperfusion injury (1–4). Aberrant Zn²⁺ regulation has also been noted in Alzheimer’s disease brain (5–7). Elevated intracellular Zn²⁺ has been associated with loss of mitochondrial membrane potential, production of reactive oxygen species, and cell death (3, 8, 9). Recently our laboratory reported that Zn²⁺ is a potent inhibitor of α-ketoglutarate-stimulated mitochondrial respiration (10). The effect of Zn²⁺ on respiration was attributed to inhibition of the α-ketoglutarate dehydrogenase complex (10). Preliminary analysis of individual subunit activities of α-ketoglutarate dehydrogenase complex indicated that the lipoamide dehydrogenase (LADH) displayed the greatest susceptibility to Zn²⁺ inhibition.

LADH belongs to the family of flavin-disulfide oxido-reductases (11), which include glutathione reductase and thioredoxin reductase. LADH is an essential component of the multienzyme NADH-generating complexes α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, branched-chain ketoacid dehydrogenase, and also glycine decarboxylase in plants (12). In these multienzyme complexes, LADH catalyzes the transfer of reducing equivalents from the bound lipote of the neighboring transferase subunit to NAD⁺.

LADH also catalyzes the reduction of free lipoic acid by NADH and thus helps to maintain the reducing environment of mitochondria required for defense against reactive oxygen species and formation of electron-rich structures such as iron-sulfur clusters. Dihydrolipoic acid, the reduced form of lipoic acid, is a potent antioxidant that scavenges reactive oxygen species such as superoxide, peroxyl radicals, hypochlorous acid, and nitric oxide. Dihydrolipoic acid may also regenerate other antioxidants such as vitamins C and E and glutathione through redox cycling (13). Preliminary human studies indicate the efficacy of lipoic acid in treatment of cerebral ischemia-reperfusion, excitotoxic amino acid brain injury, mitochondrial dysfunction, and other disorders involving free radical processes (13, 14).

LADH (EC 1.8.1.4) is a homodimeric molecule, each subunit (~52 kDa) contains two catalytic centers. FAD is responsible for NAD⁺/NADH reduction/oxidation and the redox-active disulfide interacts with the dihydrolipoyl cofactor that is covalently linked to the acetyl transferase component of each dehydrogenase complex. The reaction with lipoic acid/lipoamide is reversible. When the reaction occurs in the same direc-

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**Steady-state Kinetics**

Lipoamide Dehydrogenase Activity—All spectrophotometric measurements were performed in a 96-well plate reader (SpectraMax Plus, Molecular Dynamics, Sunnyvale, CA) with a 200-μl reaction mixture per well at 20 °C. The absorbance of NADH (ε\(_{340}\) = 6,220 m\(^{-1}\) cm\(^{-1}\) (23)) was monitored. The light path was 0.43 cm. All reactions were run in 50 mM Tris-HCl, pH 7.5. Each set of experiments was performed in triplicate. All data reported are as the means ± S.E.

Reverse reaction: the final concentrations of reagents were 30 mM LADH, 0.05–0.2 mM LA, 0.01–0.1 mM NADH, 0.1–10 μM ZnCl\(_2\). To avoid severe substrate inhibition by NADH (17) the concentration range for NADH was chosen to be lower than that for LA.

Forward reaction: the final concentrations were 30 mM LADH, 0.02–0.1 mM reduced LA, 0.2–2 mM NAD\(^+\), 1–12 μM ZnCl\(_2\) (final Me\(_3\)SO concentration in individual wells was <1%). The study of this reaction in the presence of zinc is complicated because Zn\(^{2+}\) can be coordinated by thiols, such as reduced LA and DTT. In the presence of excess DTT (1 mM) no inhibition by Zn\(^{2+}\) was observed. To avoid chelating Zn\(^{2+}\) with excess of thiol reagents, LA in Me\(_3\)SO was reduced with equimolar DTT dissolved in water and then diluted into a buffer solution saturated with argon in order to avoid oxidation. Aliquots of NAD\(^+\) and zinc solutions were placed into the wells, and the reaction was initiated by the addition of argon-saturated buffer containing reduced lipoamide and enzyme.

LADH Oxidase Activity—Oxygen consumption was measured in a "Strathkelvin Instruments" (Glasgow, UK) multichannel apparatus model SI-928 with a Clark type electrode. Reactions were monitored in thermostatted (28 °C) glass chambers fitted with special glass stoppers that contained a small injection hole (Gilson) and equipped with a magnetic stirrer ("Instech" 2060, Plymouth Meeting, PA). The electrode was calibrated in the range 0–0.240 mM oxygen immediately prior to the experiments. Oxygen concentration was varied in the range 20–240 μM by bubbling buffer with argon to displace oxygen. The final concentrations of reagents were in the range 0.1–0.2 μM enzyme, 0.01–0.05 mM NAD\(^+\), 0.01–0.25 mM NADH, and 1–40 μM ZnCl\(_2\). Data analyses were performed using "Strathkelvin Instruments" software SI version 2.1. Each experiment with the oxygen electrode was performed in triplicate.

The rate constants were calculated as an average from three independent sets of experiments.

**Transient Kinetic Studies**

Anaerobic stopped-flow measurements were performed in duplicate using a High-Tech SF-61 stopped-flow rapid scan spectrophotometer (High-Tech Scientific, Salisbury, Wiltshire, UK) installed in an anaerobic glove box operating under N\(_2\) with less than 1 ppm O\(_2\). The temperature was controlled at 22 °C with a Techné-400 circulator (Techné (Cambridge) Ltd., Duxford, Cambridgeshire, UK) with an external cooler. The enzyme solution and substrate powder were placed into the sealed serum vials and deoxygenized 1 h before being placed into the glove box. The 0.05 M Tris-HCl buffer, pH 7.5, used in all experiments was deoxygenated overnight in the glove box. NADH (0.75 mM) and ZnCl\(_2\) (1.875 mM) stock solutions were prepared anaerobically under N\(_2\) in the same buffer.

Initial experiments were performed in rapid-scan mode with a xenon lamp. The enzyme stock solution (15 M) was kept on ice. Each experiment with the oxygen electrode was performed in triplicate.

The rate constants were calculated as an average from three independent sets of experiments.
**RESULTS**

**Zn**<sup>2+</sup> Inhibition of the Reverse Lipoamide Dehydrogenase Reaction—No changes in the mechanism of LADH reaction were found within the range of 0.05–1 μM free Zn<sup>2+</sup> concentrations. The reaction continues to obey a ping-pong mechanism. In the reverse reaction, Zn<sup>2+</sup> and LA compete for one and the same enzyme form. The addition of rising concentrations of zinc results in increased slopes and an unchanged intercept in double-reciprocal plots of 1/v versus 1/[LA] (not shown). Competitive inhibition is confirmed by a clear single intersection point in the Dixon plot (see Fig. 2A), corresponding to the inhibition constant of ~0.15 μM.

With respect to NADH, Zn<sup>2+</sup> is an uncompetitive inhibitor showing parallel lines both in double-reciprocal plots and in the Dixon plot (not shown). Thus, zinc is unable to react with oxidized enzyme. The data fit the equation

\[
E_v = 1/k_{o} + 1/k_{NADH}[NADH] + 1/k_{LA}[LA](1 + [Zn]/K_{Zn})
\]

with \(k_{NADH} = (2 \pm 0.5) \times 10^8\) M<sup>-1</sup>s<sup>-1</sup>, \(k_{LA} = (3.0 \pm 0.1) \times 10^7\) M<sup>-1</sup>s<sup>-1</sup>, \(k_{o} = 300 \pm 10\) s<sup>-1</sup>, and \(K_{Zn} = 0.15 \pm 0.05\) μM.

Lipoamide and Zn<sup>2+</sup> compete for the same enzyme form. It is possible that Zn<sup>2+</sup> either attacks the charge-transfer complex, which then rearranges into a form with both thiols participating in Zn<sup>2+</sup> binding or directly binds to the form with reduced thiols, shifting the equilibrium toward the above enzyme form. We can speculate that the catalytic His residue paired with a highly conserved Glu residue also participate in Zn<sup>2+</sup> coordination (Fig. 1). The inhibition mechanism can be described by Scheme 1 with Zn<sup>2+</sup> binding the two-electron-reduced enzyme in the form of a charge-transfer complex or in the form with reduced thiols.

**Zn**<sup>2+</sup> Inhibition of the Forward Lipoamide Dehydrogenase Reaction—In the forward reaction of NAD<sup>+</sup> reduction by reduced lipoamide, Zn<sup>2+</sup> demonstrates uncompetitive inhibition with respect to lipoamide (not shown). This type of inhibition suggests that Zn<sup>2+</sup> does not react with oxidized enzyme, which is consistent with the mechanism proposed in Scheme 1. However, double-reciprocal plots of 1/v versus 1/[NAD<sup>+</sup>] indicate that Zn<sup>2+</sup> affects both slope and intercept (Fig. 2B).

If the reaction follows the mechanism presented in Scheme 1, the dependence of the forward reaction rate in double-reciprocal plots should display pure competitive inhibition with respect to NAD<sup>+</sup> in accordance with Equation 1.

\[
E_v = 1/k_{+1} + 1/k_{-3} + (k_{+3} + k_{-3}/k_{+3}/k_{o}[LA_{red}])
\]

Even if one accounts for the possible presence of oxidized lipoamide, the effect of Zn<sup>2+</sup> in accordance with Scheme 1 will be seen only on the apparent rate constant for NAD<sup>+</sup> (slope), but not on intercepts as shown in Equation 2.

\[
E_v = 1/k_{+1} + 1/k_{-3} + (k_{+3} + k_{-3}/k_{+3}/k_{o}[LA_{red}]) + (1 + [Zn]/K_{Zn})(k_{+3} + k_{-3}/k_{o}[NAD])
\]

Thus, the model presented in Scheme 1 is not consistent with the experimental data. The effect of Zn<sup>2+</sup> on both the rate constant for NAD<sup>+</sup> and the rate constant for a unimolecular step suggests that Zn<sup>2+</sup> binds to an enzyme form other than the one directly interacting with NAD<sup>+</sup>. Taking into account the existence of different forms of the two-electron-reduced enzyme (18), we can introduce a unimolecular interconversion step into Scheme 1 to get Scheme 2, which satisfactorily describes the
inhibition by Zn\(^{2+}\) for both reverse and forward reactions (see also Table I). The reverse and forward reactions exhibit equal values for the inhibition constant \(K_{\text{inhib}} = K_0(1 + k_{-1}/k_1)\), within experimental error (see Table I). The trend toward a larger inhibition constant determined for the forward reaction from Dixon plot (Fig. 2C, 0.22 ± 0.05 \(\mu\)M) compared with that determined for the reverse reaction (Fig. 2A, 0.15 ± 0.05 \(\mu\)M) may originate from Zn\(^{2+}\) chelation by reduced lipoamide.

**Zn\(^{2+}\) Interaction with the Charge Transfer Complex**—The charge transfer complex is clearly distinguishable from the oxidized enzyme and two-electron-reduced enzyme with reducing equivalents on both thiols (11) due to a characteristic shoulder at 530 nm. The effect of Zn\(^{2+}\) on the spectrum of the two-electron-reduced enzyme was studied by the stopped-flow technique under anaerobic conditions (Fig. 3). ZnCl\(_2\) addition to the charge transfer complex leads to rapid disappearance of the 530-nm shoulder and an increase in absorption at 445 nm (see inset A in Fig. 3). Thus, the Zn\(^{2+}\)-LADH complex is spectrally equivalent to the oxidized enzyme form.

The rate of the spectral change at 530 nm displays linear dependence on Zn\(^{2+}\) concentration (inset B in Fig. 3). This indicates that the experimentally measurable step is Zn\(^{2+}\) binding not interconversion between the charge-transfer complex and the dithiol form (Scheme 3). The determined rate constant for Zn\(^{2+}\) interaction with the two-electron-reduced enzyme is \((3.7 ± 0.7) \times 10^5\) M\(^{-1}\) s\(^{-1}\). This value is comparable with the rate constant for the two-electron-reduced form interaction with lipoamide determined from the steady-state kinetics \((3.0 ± 0.1) \times 10^5\) M\(^{-1}\) s\(^{-1}\). Thus, Zn\(^{2+}\) successfully competes with lipoamide for this enzyme form.

The non-zero intercept at the ordinate axis clearly points to the reversibility of the reaction and corresponds to the rate constant for the Zn\(^{2+}\)-complex dissociation (Scheme 3). The dissociation rate constant \(k_{-1}\) is equal to 0.040 ± 0.005 s\(^{-1}\). The equilibrium constant for Zn\(^{2+}\) binding calculated on the basis of stopped-flow measurements \(K_i = k_{i}/k_{-i}\) is 0.11 ± 0.04 \(\mu\)M, which is close to the inhibition constants determined from steady-state kinetics (Table I). The ratio between the inhibition constants determined from steady-state data and the binding constant determined from transient kinetics allows to estimate the ratio \(k_{-i}/K_i\) as 1.0 ± 0.5. Thus, the contribution of the charge-transfer complex and the form with reduced two thiols to the total pool of two-electron-reduced enzyme are comparable within experimental error.

**Zn\(^{2+}\) binding is reversible.** This has been demonstrated both by dilution of assay mixtures and by EDTA addition, which completely reversed all inhibitory effects (data not shown). In addition, gel-filtration of the Zn\(^{2+}\)-inhibited assay mixture restored the initial catalytic activity (results not shown).

**Zn\(^{2+}\) Stimulation of ROS Production by LADH**—Activation of molecular oxygen by flavoproteins, yielding hydrogen peroxide (dehydrogenases/transhydrogenases, oxidases) and superoxide radical (electron transferases such as flavodoxin), is a well known process (25). After the flavoenzymes of the respiratory chain, the most abundant flavoprotein in the mitochondrial matrix (−0.5% of matrix protein as calculated from Ref. 26) is lipoamide dehydrogenase. Production of hydrogen peroxide and superoxide radical catalyzed by lipoamide dehydrogenase was first reported in 1955 (27) and then confirmed in 1969 (28). More recently, identification of reaction products revealed the ratio between superoxide and hydrogen peroxide to be 1:9 (29). However, no quantitative studies to determine the oxidase reaction mechanism have been reported.

In the present work the formation of hydrogen peroxide as a major product was demonstrated by addition of catalase (Fig. 4A), which resulted in recovery of −50% of the consumed oxygen via disproportionation to \(O_2\) and \(H_2O\). Prior addition of catalase reduced the rate of oxygen consumption by half, providing further support that hydrogen peroxide is the major product (Fig. 4B). Addition of SOD at the end of the reaction resulted in detectable \(O_2\) recovery (Fig. 4, A and B) indicating the generation of superoxide anion radical, which is consistent with the published report (29).

Zn\(^{2+}\) addition increased the rate of oxygen consumption, but did not change the character or distribution of the products formed (Fig. 4A). The stimulatory effect of Zn\(^{2+}\) was observed in the presence of NAD\(^+\), although the overall reaction showed strong product inhibition (Fig. 4A). At lower oxygen concentration product inhibition by NAD\(^+\) was even more pronounced.

### Table I

| Parameter | Equation |
|-----------|----------|
| Reverse reaction | \(E_{\text{f0}} = 1/k_{-i} + 1/k_{\text{NAD}}(NADH) + 1/k_{\text{LA}}(LA)(1 + [Zn]/K_{\text{inhib}})\) |
| Equation derived for Scheme 2: | |
| First order rate constant, \(k_{-i}\) | \(k_{-i} = k_{-i}/K_{\text{inhib}}(1 + k_{-i}/k_1)\) |
| Rate constant for NADH, \(k_{\text{NADH}}\) | \(k_{\text{NADH}} = k_{-i}/K_{\text{inhib}}(1 + k_{-i}/k_1)\) |
| Rate constant for LA, \(k_{\text{LA}}\) | \(k_{\text{LA}} = k_{-i}/K_{\text{inhib}}(1 + k_{-i}/k_1)\) |
| Inhibition constant, \(K_{zi}\) | \(K_{zi} = K_{zi}(1 + k_{-i}/k_1)\) |
| Forward reaction | \(E_{\text{f0}} = 1/k_{-i}(1 + [Zn]/K_{\text{inhib}}) + 1/k_{\text{NAD}}(NAD) + 1/k_{\text{LA}}(LA)\) |
| Equation derived for Scheme 2: | |
| First order rate constant, \(k_{-i}\) | \(k_{-i} = k_{-i}/K_{\text{inhib}}(1 + k_{-i}/k_1 + k_{-i}/k_2)\) |
| Rate constant for NAD, \(k_{\text{NAD}}\) | \(k_{\text{NAD}} = k_{i}/K_{i}(1 + k_{-i}/k_1 + k_{-i}/k_2)\) |
| Rate constant for LA, \(k_{\text{LA}}\) | \(k_{\text{LA}} = k_{i}/K_{i}(1 + k_{-i}/k_1 + k_{-i}/k_2)\) |

**Experimental values:**
| Parameter | Value |
|-----------|-------|
| Inhibition constant | 0.15 ± 0.05 |
| Rate constant for NAD | 0.15 ± 0.05 |
| Rate constant for LA | 0.15 ± 0.05 |

**Steady-state kinetic parameters of Zn\(^{2+}\) inhibition of LADH reaction**
but the stimulatory effect of Zn$^{2+}$ remained clearly evident (Fig. 4C).

The dependence of the reaction rate on oxygen concentration in double-reciprocal plots shows parallel lines both in the absence and in the presence of Zn$^{2+}$ (Fig. 5A). It is also evident that Zn$^{2+}$ affects the rate constants toward NADH and oxygen in a different manner. There is little or no increase in the rate constant toward oxygen in the presence of Zn$^{2+}$ (Fig. 5A, compare slopes), while changes in the rate constant for NADH are more pronounced (Fig. 5A, see intercepts).

The study of the reaction in air-saturated buffer shows that the dependence of the initial reaction rate on NADH concentration in double-reciprocal plots is not linear (Fig. 5B). The break in slope at high concentrations of NADH clearly demonstrates a switch from one reaction mechanism to another at elevated NADH concentrations. As Zn$^{2+}$ concentration increases, the dependence on NADH concentration approaches linearity (Fig. 5B). The best fit of the data was obtained using a combination of linear and hyperbolic dependence $y = y_0 + ax/(b + x) + cx$. As Zn$^{2+}$ concentrations increased, no significant changes were observed in the parameters $y_0$, $b$, and $c$, but $a$ gradually decreases as it shown in the inset in Fig. 5B. Parameter $a$ characterizes the increase in an intercept cut by an asymptote $y_0 + a + cx$ with rising [Zn$^{2+}$].

The dependence of parameter $a$ on free [Zn$^{2+}$] is perfectly fitted to a hyperbolic dependence $a = K_{a}/K_o + [Zn^{2+}]$ with $K_o = 0.09 \pm 0.02 \mu M$ (Fig. 5B, inset). Qualitatively, the addition of Zn$^{2+}$ switches the reaction from a complex hyperbolic dependence to a simple linear dependence in double-reciprocal plots. The half-activation concentration ($K_o$) is $-0.09 \mu M$, which is close to the Zn$^{2+}$ binding constant determined from kinetic analysis of the LADH reaction. This similarity indicates the common nature of oxidase reaction activation and LADH reaction inhibition by Zn$^{2+}$.

**Mechanism of Oxidase Reaction Catalyzed by LADH**—Let us consider the NADH-oxidase reaction mechanism in the absence of Zn$^{2+}$. Upon enzyme reduction with NADH, electrons are distributed between the FAD center, which reduces oxygen to hydrogen peroxide, and the disulfide center, which does not; thus, only a portion of the two-electron-reduced enzyme is active in the oxidase reaction. To accelerate the reaction rate further, the FAD moiety must be fully reduced. A second reduction step with NADH converts the enzyme into the form containing both reduced FAD and thiols (four-electron-reduced enzyme; Scheme 4). However, the rate constant for the enzyme reduction by the second NADH molecule ($k_1$) should be much lower than that for the first one ($k_4$) because this process is less thermodynamically favorable.

To simplify the kinetic equations the rate constants $k_2$, $k_{-2}$, and $k_1$ are assumed to be equal for the left and right cycles, respectively, in Scheme 4. Assuming $k_f/k_{-3} = [E^{FADSSH2+}]/[E^{FADO2n2}] = 1$, based upon the anaerobic stopped-flow results, the dependence of initial rate on NADH, NAD$^+$, and O$_2$ is presented by Equation 3

$$E_{1/0} = 1/k_4(1 + k_1[NAD]k_1[O_2]) + 1/k_1[O_2] + (k_2[O_2] + k_1[NADH])/(k_1[O_2] + k_1[NADH])k_1[NADH]$$

(Eq. 3)

which after simple algebraic manipulations, can be presented in the form equivalent to that used for fitting the experimental data in Fig. 5B (Equation 4),

$$E_{1/0} = 1/k_4(1 + k_1[NAD]k_1[O_2]) + 1/k_2[O_2] + 1/k_1[NADH] + (k_1 - k_2)(k_2[O_2][NADH]k_1[O_2] + 1/[NADH])$$

(Eq. 4)

with $x = 1/[NADH]$ and the following expressions for the fitting parameters (Equations 5–8),

$$y_0 = 1/k_4(1 + k_1[NAD]k_1[O_2]) + 1/k_1[O_2]$$

(Eq. 5)

$$c = 1/k_4$$

(Eq. 6)

$$a = (k_1 - k_2)/k_2[O_2]$$

(Eq. 7)

$$b = k_2[O_2]$$

(Eq. 8)
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The values of \( k_1 \) and \( k_4 \) can be calculated from the fitting parameters as \( 1/c \) and \( 1/(a/b + c) \), respectively, and are equal to \((2.1 \pm 0.5) \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) and \((2.9 \pm 0.5) \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) in the absence of Zn\textsuperscript{2+}.

**Mechanism of Oxidase Reaction in the Presence of Zn\textsuperscript{2+}**—It can be assumed that all enzyme forms containing catalytic dithiols are capable of binding Zn\textsuperscript{2+} ion (Scheme 5). Zn\textsuperscript{2+} binding switches the rate for the NADH interaction from the slow two-electron-reduced enzyme rate (\( k_c \)) to a much faster rate (\( k'_c \)). We again assume that \( k_{3b} \), \( k_{-3b} \), and \( k_4 \) are equal for all enzyme forms, respectively. The equilibrium between Zn\textsuperscript{2+}-bound and free thiol-reduced enzyme forms is characterized by an apparent activation constant \( K_a \). Under the above conditions, a simplified analytical treatment of the above scheme under the conditions of \( k'_c [\text{NADH}] \gg k_c [O_2] \) gives the following expression for the initial reaction rate (Equation 9).

\[
E_{\text{rate}} = k_1 [NAD] + k_2 [O_2] + [Zn][k_c][NAD] + [k_c][O_2] + k_3 [NAD][H][k][O_2] + k_4 [NAD][H][k][NAD][1 + [Zn][K_a]] \quad (\text{Eq. 9})
\]

The above equation corresponds to Equation 4 and the addition of a Zn\textsuperscript{2+}-dependent factor \( 1/(1 + [Zn]/K_a) = K_a/[K_a + [Zn]] \) to the hyperbolic term. This exactly describes the experimental dependence obtained for parameter \( a_{zm} = ([k_c - k_c][k_c][O_2])_{Zn=0} = ([k_c - k_c][k_c][O_2])_{Zn=0} K_a/[K_a + [Zn]] \) (Fig. 5B, inset). At [Zn] \( \to 0 \) Equation 9 converts to Equation 4, and at [Zn] \( \to \infty \) the NADH dependence presented by Equation 9 converts into a linear one (Equation 10), which is consistent with the experimental data.

\[
E_{\text{rate}} = 1/k_c [1 + k_c [NAD][O_2] + [k_c][O_2] + [k_c][NAD][H][1 + [Zn][K_a]]] \quad (\text{Eq. 10})
\]

The value of \( k'_c \) extracted from the fitting parameters in the presence of Zn\textsuperscript{2+} (\( k'_c = (4.4 \pm 0.5) \times 10^4 \text{ M}^{-1} \text{s}^{-1} \)) is two times higher than \( k_c = (2.1 \pm 0.5) \times 10^4 \text{ M}^{-1} \text{s}^{-1} \). This may indicate better FAD accessibility for NADH in the Zn\textsuperscript{2+}-modified enzyme.

The mechanistic approach developed to describe LADH behavior in the oxidase reaction is consistent with the experimental data. A number of kinetic parameters, characteristic of individual catalytic steps, can be determined from fitting parameters. The apparent binding constant characterizing the interaction of Zn\textsuperscript{2+} with the pool of thiol-reduced enzyme forms...
was determined as $K_a = 0.09 \pm 0.02 \mu M$, which is in agreement with the value determined from stopped-flow data ($K_a = 0.11 \pm 0.02 \mu M$). The kinetic parameters of the oxidase reaction are summarized in Table II.

Thus, the activation effect on the LADH-catalyzed oxidase reaction by Zn$^{2+}$ is mainly due to a switch from the rate constant for the reduction of two-electron-reduced enzyme by the second NADH molecule ($k_5 = (3.4 \pm 0.4) \times 10^9 \text{M}^{-1} \text{s}^{-1}$) to the rate constant for the reduction of Zn$^{2+}$-modified enzyme with NADH ($k_5' = (4.4 \pm 0.5) \times 10^9 \text{M}^{-1} \text{s}^{-1}$). This change results in a 4- to 5-fold increase of the steady-state LADH catalytic activity in the oxidase reaction in the presence of saturating Zn$^{2+}$ and buffer equilibrated with atmospheric oxygen.

**NAD$^+$ Inhibition of the Oxidase Reaction**—The mechanistic approach developed above provides a framework to study the effect of product, NAD$^+$, on the reaction mechanism. Earlier work (29) noted in passing that NAD$^+$ strongly inhibited the oxidase reaction. We observed that in the presence of saturating Zn$^{2+}$ and atmospheric oxygen concentrations the enzyme shows no inhibition by NAD$^+$ up to 0.02 mM concentrations, while in the absence of Zn$^{2+}$ the enzyme is strongly inhibited (compare Fig. 6 A and B). Above this threshold NAD$^+$ inhibits the reaction in the presence of Zn$^{2+}$, as well (Fig. 6B). Fitting of the data to Equations 4 and 9 allows determination of $k_1$ and $k_1'$, respectively. While the value of $k_1'$ is the same as the one extracted from the data presented in Fig. 6A, the value of $k_1$ is smaller by a factor of 2, (compare $k_1' = (4.4 \pm 0.5) \times 10^9 \text{M}^{-1} \text{s}^{-1}$ and $k_1 = (2.1 \pm 0.5) \times 10^9 \text{M}^{-1} \text{s}^{-1}$ determined from Fig. 5B) but of 3.5 and is equal to $(3.2 \pm 0.2) \times 10^9 \text{M}^{-1} \text{s}^{-1}$. The confidence in the second determination is higher because it relies upon fitting to a larger data set (six titration curves in Fig. 6A) than the original determination (Fig. 6A, no Zn$^{2+}$).

As predicted from Equations 4 and 9, and actually observed, the presence of NAD$^+$ affects parameter $y_0 = 1/k_4(1 + k^{-2}_2[NAD]/k_4[O_2])$ in a linear manner. Dixon plots (Fig. 6 C and D) of the data presented in Fig. 6, A and B confirm the linear character of NAD$^+$-inhibition with an apparent inhibition constant of $\sim 70 \mu M$. Based on this value and the value for $k_4$, the value $k^{-2}_2$ can be calculated as $10^4 \text{M}^{-1} \text{s}^{-1}$ (Table II).

NAD$^+$ inhibition in the absence of Zn$^{2+}$ (Fig. 6C) is nearly competitive with respect to NADH. This suggests competition of NAD$^+$ and NADH for the same enzyme form, i.e. the two-electron-reduced enzyme. This result is consistent with the mechanism presented in Scheme 5. In the presence of saturating Zn$^{2+}$ and low [NADH], NAD$^+$ inhibits the reaction in an uncompetitive manner (Fig. 6D) and thus, NADH and NAD$^+$ compete for different enzyme forms. In other words, under these conditions the enzyme is distributed between the left and right cycles in Scheme 5. At higher [NADH], NAD$^+$ inhibits the reaction in a competitive manner indicating the involvement of the bottom right cycle in Scheme 5.

The observed degree of NAD$^+$ inhibition strongly depends on available oxygen (the ratio between $k^{-2}_2[NAD]$ and $k_4[O_2]$), see Equations 4 and 9). Under the conditions of low oxygen, the inhibition by NAD$^+$ should be more severe, which is consistent with the results obtained at 30 $\mu M$ oxygen (see Fig. 4C). Thus, in the presence of NAD$^+$ and low oxygen, ROS production by LADH will be completely suppressed, while the presence of Zn$^{2+}$ will switch the reaction on.

**DISCUSSION**

The current study of LADH-catalyzed lipoamide dehydrogenase and oxidase reactions provides evidence for strong effects of submicromolar free Zn$^{2+}$ on the catalytic activity of this important enzyme of energy metabolism. Internalization of cytosolic Zn$^{2+}$ into the mitochondrial matrix has not been unequivocally demonstrated. However, indirect evidence was provided for isolated mitochondria by monitoring disappearance of extra-mitochondrial Zn$^{2+}$ and competition with Ca$^{2+}$ transport.
Zn\(^{2+}\) Stimulates ROS Production by LADH

(30). Zn\(^{2+}\) inhibition of respiration in isolated, intact mitochondria in a substrate-dependent manner (10) provides further indirect evidence of Zn\(^{2+}\) internalization by mitochondria.

The inhibition of the forward lipoamide dehydrogenase reaction suggests that elevated Zn\(^{2+}\) concentrations inside the mitochondrial matrix will inhibit NADH production by the keto-glutarate, pyruvate, and branched-chain dehydrogenases, interrupting the Krebs cycle and mitochondrial energy metabolism. LADH inhibition may be sufficient to explain the previously observed selectivity of Zn\(^{2+}\) inhibition for \(\alpha\)-keto-glutarate-dependent respiration over glutamate/malate or succinate-dependent respiration in intact mitochondria (10).

Zn\(^{2+}\) inhibition of lipoamide dehydrogenase activity in the reverse reaction may also have important physiological consequences. Elevated intra-mitochondrial Zn\(^{2+}\) concentrations would interfere with dihydrolipoate recycling catalyzed by LADH. Interference with reduced thiol recycling could underlie the mechanism of Zn\(^{2+}\) induction of permeability transition (31), which is sensitive to thiol redox status (32).

Zn\(^{2+}\) is known to stimulate a burst of mitochondrial ROS production (9). The conventional model is that this is due to a blockade of the electron transport chain at the level of Complex III. It has been demonstrated that cytochrome b\(_c\) is inhibited by Zn\(^{2+}\), and two Zn\(^{2+}\) binding sites have been recently localized in the crystal structure (33). Inhibition of b\(_c\) by antimycin A results in increased generation of superoxide, which is converted to \(H_2O_2\) (Refs. 34, 35 and references therein). As demonstrated above, LADH oxidase activity produces \(H_2O_2\) and superoxide directly and is strongly induced by Zn\(^{2+}\). A preliminary estimate of LADH-catalyzed oxidase activity in mitochondria can be made using the reported value of LADH activ-ity in the rat liver mitochondrial matrix fraction (26) and the rate constants determined in this work (Table II). In the presence of 0.1 \(\mu\)M NADH, saturating oxygen, saturating Zn\(^{2+}\), and at 37 \(\degree\)C, mitochondrial LADH could produce \(\sim10\) nmol of \(H_2O_2\) per min per mg of matrix protein. This corresponds to \(\sim1\) nmol/min per mg of total mitochondrial protein (compared with antimycin A-induced mitochondrial production of 2.5 nmol of \(H_2O_2\) per min/mg of protein at 37 \(\degree\)C (36)). Thus, it is possible that under some pathological conditions LADH may contribute significantly to the overall ROS load of mitochondria.

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APPENDIX

Background—Under the equilibrium conditions with Tris and enzyme competing for Zn\(^{2+}\), the system is described by the following equilibria,

\[ K_{Tris} = [\text{Tris}][Zn]/[\text{TrisZn}] \]

\[ K_{Zn} = [E][Zn]/[EZN] \]

and three equations of material balance,

\[ [\text{Zn}] = [\text{Zn}] + [\text{TrisZn}] + [EZN] \]

\[ [E] = [E]_0 + [EZN] \]

\[ [\text{Tris}] = [\text{Tris}]_0 + [\text{TrisZn}] \]

where \([E]_0\), \([\text{Zn}]_0\), and \([\text{Tris}]_0\) are the initial concentrations of enzyme, Zn\(^{2+}\), and Tris, \([E]\), \([\text{Zn}]\), and \([\text{Tris}]\) are their free and bound concentrations, respectively. Under the experimental conditions with Zn\(^{2+}\) concentrations much lower than Tris concentration \(([\text{Zn}]_0 < < [\text{Tris}]_0\)) the concentration of Tris-bound Zn\(^{2+}\) is also much smaller than Tris concentration \(([\text{TrisZn}] << [\text{Tris}]_0\)) and the binding constant can be approximated as follows.

\[ K_{Tris} = ([\text{Tris}]_0 - [\text{TrisZn}])/[\text{TrisZn}] = [\text{Tris}]_0/[\text{TrisZn}] \]

If the total enzyme concentration is much lower than the total concentration of Zn\(^{2+}\), the term \([EZN]\) can be ignored in the equation of material balance since \([EZN] < [E]_0 << [\text{Zn}]_0\). Therefore,

\[ [\text{Zn}] = [\text{Zn}] + [\text{Tris}]/K_{Tris} \]

Rearranging, the concentration of free Zn\(^{2+}\) available for en-zyme binding can be expressed as follows.

\[ [\text{Zn}] = [\text{Zn}]/(1 + [\text{Tris}]/K_{Tris}) \]

Thus, the knowledge of zinc binding constant by Tris is sufficient to calculate available free zinc concentration. The determination of \(K_{Tris}\) under the conditions used in the kinetic experiments, i.e., 50 \(mM\) Tris-HCl, pH 7.5, was performed using the Zn\(^{2+}\)-selective fluorescent indicator APTRA-BTC, tripotas-sium salt (Molecular Probes, Eugene, OR).

To determine the constant for Tris-Zn binding in the presence of dye we consider the same equations as above. Replacing the enzyme, \(E\), with the dye, \(B\), we have the following expression for the ratio of the binding constants.

\[ K_{Tris} = [\text{Tris}]/(1 + [\text{Tris}]/K_{Tris}) \]

and thus,

\[ K_{Tris} = K_{B}[B]/([\text{Tris}]/K_{Tris}). \]

Working under the conditions \([B]_0 << [\text{Zn}]_0 << [\text{Tris}]_0\) we can assume \([\text{Tris}] = [\text{Tris}]_0\) and \([\text{TrisZn}] = [\text{Zn}]_0\). In this case \(K_{Tris} = K_{B}B/[\text{Zn}]_0\).
For simplicity, we have employed a factor of 20 to calculate free Zn$^{2+}$ concentrations.

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$K_{\text{rec}} = K_{\text{APTRA}} / K_{\text{Zn^2+}}$. For simplicity, we have employed a factor of 20 to calculate free Zn$^{2+}$ concentrations.