Zn\textsuperscript{2+} Inhibits α-Ketoglutarate-stimulated Mitochondrial Respiration and the Isolated α-Ketoglutarate Dehydrogenase Complex*  

Received for publication, August 23, 1999, and in revised form, January 4, 2000

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The pool of cellular Zn\textsuperscript{2+} that is not tightly bound to macromolecules or to other ligands and can be readily chelated by Zn\textsuperscript{2+}-sensitive chromophores or fluorophores has been termed “chelatable zinc” (1). Interest in the biological function of chelatable zinc has grown steadily during the last decade. This interest was stimulated in part by the recognition that the concentration of Zn\textsuperscript{2+} is elevated in a variety of pathological conditions, including ischemia-reperfusion injury and Alzheimer’s disease. Impairment of mitochondrial respiration is also associated with these pathological conditions. To test whether elevated Zn\textsuperscript{2+} and impaired respiration might be linked, respiration of isolated rat liver mitochondria was measured after addition of Zn\textsuperscript{2+}. Zn\textsuperscript{2+} inhibition (K\textsuperscript{app} = 1 M) was observed for respiration stimulated by α-ketoglutarate at concentrations well within the range of intracellular Zn\textsuperscript{2+} reported for cultured hepatocytes. The bc\textsubscript{1} complex is inhibited by Zn\textsuperscript{2+} (Link, T. A., and von Jagow, G. (1995) J. Biol. Chem. 270, 25001–25006). However, respiration stimulated by succinate (K\textsuperscript{app} = 6 M) was less sensitive to Zn\textsuperscript{2+}, indicating the existence of a mitochondrial target for Zn\textsuperscript{2+} upstream from bc\textsubscript{1} complex. Purified pig heart α-ketoglutarate dehydrogenase complex was strongly inhibited by Zn\textsuperscript{2+} (K\textsuperscript{app} = 0.37 ± 0.05 M). Glutamate dehydrogenase was more resistant (K\textsuperscript{app} = 6 M), malate dehydrogenase was unaffected, and succinate dehydrogenase was stimulated by Zn\textsuperscript{2+}. Zn\textsuperscript{2+} inhibition of α-ketoglutarate dehydrogenase complex required enzyme cycling and was reversed by EDTA. Reversibility was inversely related to the duration of exposure and the concentration of Zn\textsuperscript{2+}. Physiological free Zn\textsuperscript{2+} may modulate hepatic mitochondrial respiration by reversible inhibition of the α-ketoglutarate dehydrogenase complex. In contrast, extreme or chronic elevation of intracellular Zn\textsuperscript{2+} could contribute to persistent reductions in mitochondrial respiration that have been observed in Zn\textsuperscript{2+}-rich diseased tissues.

The pool of cellular Zn\textsuperscript{2+} that is not tightly bound to macromolecules or to other ligands and can be readily chelated by Zn\textsuperscript{2+}-sensitive chromophores or fluorophores has been termed “chelatable zinc” (1). Interest in the biological function of chelatable zinc has grown steadily during the last decade. This interest was stimulated in part by the recognition that the concentration of chelatable Zn\textsuperscript{2+} is elevated in some cerebral regions following episodes of transient ischemia (2, 3) or excitotoxic injury (4). Elevated intracellular Zn\textsuperscript{2+} has also been observed in models of cardiac ischemia and cardiac inflammation (5, 6). Excess intracellular Zn\textsuperscript{2+} is toxic to neurons (7–9). Various mechanisms for the toxic activity of Zn\textsuperscript{2+} have been proposed including modulation of amino acid receptor activity (10–13), alteration of nerve growth factor binding (14), induction of gene expression (15), and alterations of mitochondrial function (7, 8). Elevated intracellular free Zn\textsuperscript{2+} and reduced carbohydrate flux through mitochondrial energy pathways (16–21) are prominent pathological features in some neurological and cardiac diseases.

Oxidative energy metabolism is impaired in many neurodegenerative disorders (22–24). One component of energy metabolism that has been extensively studied is the α-ketoglutarate dehydrogenase complex (KGDHC). Reductions in KGDHC activity or protein abundance in brain occur in several neurological diseases (25–30). The cause of this deficit has not been established. Reduced concentration and/or activity of enzymes involved in mitochondrial carbohydrate metabolism has been documented for both chronic ischemia and ischemia-reperfusion injury in heart and brain (20, 21, 31).

Studies on intact mitochondria have revealed that Zn\textsuperscript{2+} inhibits respiration supported by combined glutamate and malate (32) or β-hydroxybutyrate (33). Subsequent studies identified complex III, specifically the bc\textsubscript{1} complex, as the site of Zn\textsuperscript{2+} binding and inhibition (34, 35). (Similarly, cytochrome b<sub>562</sub>-o complex of aerobically grown Escherichia coli K12 is inhibited by Zn\textsuperscript{2+} (36).) These studies utilized substrates that enter the respiratory chain downstream from complex I such as succinate, nonylubihydroquinone, or duroquinol (32, 34, 35).

The choice of substrates in the earlier studies precluded detection of Zn\textsuperscript{2+} inhibition of upstream dehydrogenases. Evidence of disease-linked impairment of KGDHC activity (see previous paragraph), which is part of the (Krebs) tricarboxylic acid cycle that is upstream from complex I, motivated us to examine the effect of Zn\textsuperscript{2+} on earlier stages of mitochondrial energy metabolism. Because the cytosolic concentration of free Zn\textsuperscript{2+} in dissociated hepatocytes is in the range of 0.6–2.7 M (37), we tested the effect of submicromolar to micromolar Zn\textsuperscript{2+} upon mitochondrial respiration.

KGDHC converts α-ketoglutarate (α-KG), coenzyme A (CoA), and NAD<sup>+</sup> to succinyl-CoA, CO₂, and NADH in the presence of

* This work was supported by National Institutes of Health (NIH)/NINDS Grant NS38741 (to A. M. B.). Pilot support was provided from Leadership and Excellence Award in Alzheimer’s Disease AG09014 NIH/NIA (to J. P. B.) and the Winifred Masterson Burke Relief Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: KGDHC, α-ketoglutarate dehydrogenase complex; α-KG, α-ketoglutarate; BSA, bovine serum albumin; CoA, coenzyme A; CoASH, coenzyme A, reduced form; DTT, dithiothreitol; E1, α-ketoglutarate dehydrogenase; E2, dihydrolipoyl acyltransferase; E3, dihydrolipoamide dehydrogenase (lipoamide dehydrogenase); GDH, glutamate dehydrogenase; MDH, malate dehydrogenase; LipS<sub>2</sub>, lipoylmoide, oxidized form; Lip[SH]<sub>2</sub>, reduced lipoamide (=dihydrolipoamide); SDH, succinate dehydrogenase; TPP, thiamin pyrophosphate.
Zn$^{2+}$ Inhibition of Mitochondrial KGDHC

thiamin pyrophosphate (TPP) (38). The complex subunit structure of KGDHC has been extensively studied. The sequential activities catalyzed by KGDHC (39) are summarized below. KGDHC activity is feedback-inhibited by the reaction products NADH and succinyl-CoA (40). KGDHC is also sensitive to metals; activity is enhanced by millimolar Mg$^{2+}$ or micromolar Ca$^{2+}$ (41) but is inhibited by higher (>20 mM) concentrations of Ca$^{2+}$ (42, 43).

In this paper, we report that micromolar concentrations of Zn$^{2+}$ in the assay buffer inhibit respiration of intact liver mitochondria. Respiration stimulated by α-KG is more sensitive to Zn$^{2+}$ inhibition than respiration stimulated by other mitochondrial energy substrates. Experiments with purified enzyme indicated that KGDHC is inhibited by submicromolar concentrations of Zn$^{2+}$. The possible roles of Zn$^{2+}$ in the regulation of mitochondrial respiration and in mitochondrial pathology are discussed.

MATERIALS AND METHODS

Reagents—Sodium α-KG, sodium succinate, l-glutamate, malate, NADH, NAD$^+$, TPP, Tris, CoASH, diithiothretol (DTT), fatty acid-free bovine serum albumin (BSA; A-6063, 10 g/l), lipoamide (LipS$_2$), 2,6-dichloroindophenol, p-iodonitrotetrazolium violet, EDTA, ZnCl$_2$, CaCl$_2$, and MgCl$_2$ were purchased from Sigma. Enzyme activities are expressed as nmoles of product formed per min at 37 °C.

Preparation of KGDHC—The enzyme is sensitive to inhibition by low concentrations of Zn$^{2+}$ (see “Results”). Therefore, as a prerequisite for the study of the effect of Zn$^{2+}$ on KGDHC, the purified enzyme complex was subjected to Sephadex chromatography to remove EDTA, E3, and BSA (components of the storage buffer that chelate Zn$^{2+}$ and other divalent ions). An aliquot (100 μl) of the commercial KGDHC preparation was loaded onto a 0.5 × 8 cm Sephadex G-200 (Amersham Pharmacia Biotech) column equilibrated with 50 mM Tris-HCl, pH 7.4, and 0.1 mM DTT. The enzyme was eluted with 100-μl aliquots of the same buffer, and fractions containing enzyme activity (fractions 6–8) were used. Activity of the most-concentrated fractions was stable at 4 °C for 3–7 days.

Enzyme and Subunit Reaction Schemes—KGDHC is composed of multiple copies of three different subunits that transfer intermediate enzyme products in an ordered fashion (39). The combined E1, E2, and E3 subunits of KGDHC catalyze the following reaction sequence in the presence of α-KG, TPP, CoA, and NAD$^+$. α-KG + TPP-E1 → succinyl-TPP-E1 + CO$_2$ (Eq. 1)

Succinyl-TPP-E1 + [LipS$_2$]E2 → succinyl-[SLipSH]E2 + TPP-E1 (Eq. 2)

Succinyl-[SLipSH]E2 + CoASH → [LipSH]$_2$E2 + succinyl-SCoA (Eq. 3)

[Lip(SH)$_2$]E2 + E3-FAD → [LipS$_2$]E2 + Reduced E3-FAD (Eq. 4)

Reduced E3-FAD + NAD$^+$ → E3-FAD + NADH + H$^+$ (Eq. 5)

where [Lip(SH)$_2$] and [Lip$_2$] represent the dihydrolipoamide (reduced form) and lipoamide (oxidized form) of the tethered lipoic acid prosthetic group of E2, respectively (39). The overall reaction is as follows.

α-KG + CoASH + NAD$^+$ → Succinyl-SCoA + NADH + H$^+$ + CO$_2$ (Eq. 6)

Assay of the combined E1-E2 activity (Equations 1–3) can be accomplished by introducing free LipS$_2$ to replace E3-FAD as an electron acceptor (Equation 7). The reduced lipoamide prosthetic group is oxidized by disulfide exchange, allowing E2 to recycle while generating a pool of Lip(SH)$_2$.[Lip(SH)$_2$]E2 + LipS$_2$ → [LipS$_2$]E2 + Lip(SH)$_2$ (Eq. 7)

Subsequent quaternation of Lip(SH)$_2$ is accomplished in an end point assay that measures the burst of NADH produced upon addition of the accumulated Lip(SH)$_2$ to a mixture of purified E3 and excess NAD$^+$. Lip(SH)$_2$ + E3-FAD → Lip$_3$ + reduced E3-FAD (Eq. 8)

Reduced E3-FAD then converts NAD$^+$ to NADH (Equation 5) giving the following overall reaction.

Lip(SH)$_2$ + NAD$^+$ → Lip$_3$ + NADH + H$^+$ + CO$_2$ (Eq. 9)

Assay of E3 activity (independent of E1 and E2) is accomplished by providing exogenous Lip(SH)$_2$ and NAD$^+$ to either KGDHC or isolated E3, as in Equation 9. Reaction 9 is freely reversible. Therefore, the reverse (disphosphorylation) reaction catalyzed by E3 can be monitored in the presence of NADH and LipS$_2$ (see below).

Activity Measurements of KGDHC and Its Components—The volume of the reaction mixtures was 200 μl except where noted. Activities were determined spectrophotometrically by using BSA as a standard (47). Mitochondrial Respiration Assays—Mitochondrial oxygen consumption was measured at 28 °C using a Clark electrode in a computer controlled system (Hansatech, PP Systems, Haverhill, MA) as described previously (48). Mitochondria were suspended in buffer A (250 mM mannitol, 75 mM sucrose, 10 mM HEPES, adjusted to pH 7.4 with KOH) supplemented with 100 μM K-EGTA and 0.5% (w/v) fatty acid-free BSA (final mitochondrial concentration, 0.8 mg/ml of protein; 115 μM Ca$^{2+}$, 500 μM K-EGTA, and 0.5% (w/v) fatty acid-free BSA. Following the final wash, mitochondria were re-suspended in buffer A and 5 μM K-EGTA. Protein concentrations were estimated spectrophotometrically using BSA as a standard (47). Mitochondrial Respiration Assays—Mitochondrial oxygen consumption was measured at 28 °C using a Clark electrode in a computer controlled system (Hansatech, PP Systems, Haverhill, MA) as described previously (48).mitochondria were suspended in buffer A (250 mM mannitol, 75 mM sucrose, 10 mM HEPES, adjusted to pH 7.4 with KOH) supplemented with 100 μM K-EGTA and 0.5% (w/v) fatty acid-free BSA (final mitochondrial concentration, 0.8 mg/ml of protein; 115 μM Ca$^{2+}$, 500 μM K-EGTA, and 0.5% (w/v) fatty acid-free BSA. Following the final wash, mitochondria were re-suspended in buffer A and 5 μM K-EGTA. Protein concentrations were estimated spectrophotometrically using BSA as a standard (47).
Materials and Methods. Zn^{2+} concentrations are as indicated. The arrow indicates the addition of ADP to initiate state 3 respiration. Mitochondria were incubated with Zn^{2+} for 3 min prior to initiation of state 3. A. Respiration in the presence of succinate. B, respiration in the presence of glutamate/malate couple. C, respiration in the presence of α-KG.

Data Analyses—All data are reported as the means ± S.E. Enzyme velocities (V) were determined by regression analysis of the change in absorbance at 540 nm. Mitochondria were preincubated for 2 min in the presence of 0–6.4 μM Zn^{2+}. Substrate was added, and 3 min of state 4 respiration was monitored prior to ADP addition (state 3 respiration). Fig. 1A illustrates that in the presence of succinate state 3 respiration was at least partly maintained at 6.4 μM Zn^{2+}. All five mitochondrial preparations tested in the presence of succinate failed to recover state 4 respiration when exposed to 6.4 μM Zn^{2+}, which may be due to uncoupling and/or ATPase activation. Glutamate/malate-stimulated state 3 respiration was somewhat inhibited by 0.4 or 1.6 μM Zn^{2+} and completely inhibited by 6.4 μM Zn^{2+} (Fig. 1B). In contrast, partial inhibition of α-KG-stimulated State 3 respiration was observed at 0.4 μM, and complete inhibition required only 3.2 μM Zn^{2+} (Fig. 1C). The IC_{50} values for each substrate (Table I) were statistically different from each other (p < 0.05). The K_{i}^{app} for Zn^{2+} inhibition of respiration determined analytically (Fig. 2) also differed significantly for each substrate and was smallest for α-KG (Table I).

Effect of Zn^{2+} on SDH, GDH, and MDH—The above findings suggested that there might be previously unidentified sites in the respiratory chain that are upstream from and more sensitive to Zn^{2+} inhibition than the bc_{1} complex. Possible sites of Zn^{2+} inhibition include mitochondrial dehydrogenases associated with the oxidation of the various metabolites tested above. Therefore, the Zn^{2+} sensitivities of the relevant mitochondrial dehydrogenases were directly tested.

To obtain additional evidence in support of the proposed mechanism of Zn^{2+} action, the effect of Zn^{2+} on purified KGDHC was carried out.

RESULTS

Effect of Zn^{2+} on Mitochondrial Respiration—Respiration was measured in isolated, intact liver mitochondria by monitoring oxygen consumption using succinate, glutamate/malate, or α-KG as substrates. Note that 3 mM Mg^{2+} was included in the respiration buffer to protect mitochondria from induction of the permeability transition (46, 50, 51). Under these conditions Zn^{2+} did not induce mitochondrial swelling, as assessed by absorbance at 540 nm. Mitochondria were preincubated for 2 min in the presence of 0–6.4 μM Zn^{2+}. Substrate was added, and 3 min of state 4 respiration was monitored prior to ADP addition (state 3 respiration). Fig. 1A illustrates that in the presence of succinate state 3 respiration was at least partly maintained at 6.4 μM Zn^{2+}. All five mitochondrial preparations tested in the presence of succinate failed to recover state 4 respiration when exposed to 6.4 μM Zn^{2+}, which may be due to uncoupling and/or ATPase activation. Glutamate/malate-stimulated state 3 respiration was somewhat inhibited by 0.4 or 1.6 μM Zn^{2+} and completely inhibited by 6.4 μM Zn^{2+} (Fig. 1B). In contrast, partial inhibition of α-KG-stimulated State 3 respiration was observed at 0.4 μM, and complete inhibition required only 3.2 μM Zn^{2+} (Fig. 1C). The IC_{50} values for each substrate (Table I) were statistically different from each other (p < 0.05). The K_{i}^{app} for Zn^{2+} inhibition of respiration determined analytically (Fig. 2) also differed significantly for each substrate and was smallest for α-KG (Table I).

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Preparation of Chelator-free KGDHC—Preliminary experiments with purified KGDHC indicated that Zn^{2+} in the range of 2–5 μM inhibited enzyme activity. However, the value of the determined K_{i}^{app} progressively declined as the concentration of enzyme in the reaction mixture was reduced (data not shown).
Zn\(^{2+}\) Inhibition of Mitochondrial KGDHC

The enzyme storage buffer contains EDTA and EGTA, both highly avid chelators of Zn\(^{2+}\) (52), and BSA, which has been reported to bind Zn\(^{2+}\) with submicromolar affinity (53). Direct dilution of KGDHC in storage buffer into assays results in concentrations of 2–5 \(\mu\)M for both EDTA and EGTA and 0.1–0.3 \(\mu\)M BSA in the final assay mixture. Therefore, the residual concentration of chelators was high enough to interfere with the determination of inhibition constants for metal ions. EDTA, EGTA, and BSA were simultaneously removed from the enzyme preparation by rapid gel filtration chromatography on a Sephadex 200 column. As expected, the apparent \(K_{\text{app}}\) for Zn\(^{2+}\) decreased after these chelators were removed from the enzyme stock. Denaturing gel electrophoresis stained with Coomassie Blue confirmed that G-150 chromatography removed >97% of the BSA initially found in the enzyme preparation (data not shown). KGDHC subjected to gel filtration was used in all of the experiments presented below.

**Inhibition of KGDHC Activity by Zn\(^{2+}\) Is Concentration-dependent**—Fig. 4A illustrates the inhibition of the KGDHC reaction by Zn\(^{2+}\) in the presence of saturating substrates and co-factors. Zn\(^{2+}\) addition to KGDHC results in a dose-dependent slowing in the rate of reaction product formation (Fig. 4A). The slow onset of inhibition causes a notable curvature in the reaction progress curves, presenting a dilemma in the assignment of velocity values used in the determination of \(K_{\text{app}}\). In the experiments described below a standard interval of 15 min was arbitrarily chosen for calculating velocity (\(V_{\text{av}}\)). Dixon plots of \(1/V_{\text{av}}\) versus the Zn\(^{2+}\) concentration for three different enzyme concentrations (Fig. 4B) resulted in similar intercepts on the x-axis, providing an estimate of \(-\frac{1}{K_{\text{app}}^{\text{enzyme}}}\) (49). The lack of dependence of \(K_{\text{app}}^{\text{enzyme}}\) on enzyme concentration indicates the successful removal of chelators. The average \(K_{\text{app}}^{\text{enzyme}}\) for Zn\(^{2+}\) for four preparations of chelator and BSA-free KGDHC was 0.37 ± 0.05 \(\mu\)M.

The sensitivity of KGDHC to inhibition by low concentrations of Zn\(^{2+}\) raised the possibility that KGDHC might be inhibited by residual Zn\(^{2+}\) or another metal in an assay mixture that is free of chelators. Fig. 4C demonstrates that \(V_{\text{av}}\) increases upon 2-fold upon addition of EDTA to the assay reaching a plateau at ~0.3 \(\mu\)M EDTA. The source of this metal has not been determined.

**Inhibition of KGDHC Activity by Zn\(^{2+}\) Requires Enzyme Cycling**—The gradual onset of inhibition by Zn\(^{2+}\), reflected by curvature during the first minutes of the reaction (Fig. 4A), is consistent with a slow binding mechanism (54). Preincubation of KGDHC with Zn\(^{2+}\) might be expected to allow the slow binding step to take place prior to initiation of the enzyme reaction. However, preincubation of KGDHC with Zn\(^{2+}\) in the presence of all substrates except \(\alpha\)-KG for 78 min did not abolish the curvature of the reaction progress curves (data not shown). This observation indicates that substrate cycling in the presence of Zn\(^{2+}\) is required for the development of inhibition of KGDHC.

The requirement for substrate cycling in the inhibition of KGDHC is further illustrated by the data in Table II. Preincubation of KGDHC with Zn\(^{2+}\) for up to 78 min did not change the value of \(K_{\text{app}}\) when \(V_{\text{av}}\) was determined from the initial 15-min interval that followed addition of \(\alpha\)-KG. In contrast, \(K_{\text{app}}\) declined steadily when \(V_{\text{av}}\) was determined for intervals that were sampled after increasing periods of substrate cycling (Table II). These data indicate that after about 1 h of cycling the calculated \(K_{\text{app}}\) was reduced by about one half.

**Partial Reversibility of Inhibition of KGDHC by Zn\(^{2+}\)**—The KGDHC-catalyzed reactions represented in Fig. 4A were allowed to proceed in the presence of 0–5 \(\mu\)M Zn\(^{2+}\) for 110 min. At the end of this period, each reaction mixture was adjusted to a final concentration of 10 \(\mu\)M EDTA, and the reaction progress was again monitored (Fig. 5A). A gradual increase in enzyme activity was apparent. Control experiments indicated that 10 \(\mu\)M EDTA was sufficient to attain maximal reversal of KGDHC inhibition for Zn\(^{2+}\) concentrations up to 5 \(\mu\)M (data not shown). However, the extent of reversal of KGDHC inhibition was dependent upon the prior concentration of free Zn\(^{2+}\). This effect was manifested in two ways: the time to reach a linear rate of product formation was greater, and the maximal rate of product formation was lower for samples that were previously exposed to higher Zn\(^{2+}\) (e.g., compare 0.5 and 5 \(\mu\)M traces in Fig. 5A).

The extent of KGDHC recovery was also dependent upon the duration of exposure to Zn\(^{2+}\). Fig. 5B shows the recovery for reaction mixtures that were exposed to the same concentration of Zn\(^{2+}\) (1 \(\mu\)M) for different reaction durations prior to reversal by EDTA. The time to reach maximum reaction velocity and the magnitude of this velocity after EDTA reversal diminished as the duration of exposure to a fixed Zn\(^{2+}\) increased.

### Table I

| Substrate               | \(n\) | 
|------------------------|------|
| \(\alpha\)-Ketoglutarate| 5    |
| Succinate              | 5    |
| Glutamate/malate       | 4    |

### Footnotes

1. Dixon plot calculated from Fig. 2.
2. \(p = 0.0007\) versus succinate, paired t test; \(p = 0.040\) versus glutamate/malate, paired t test.
3. \(p < 0.0084\) versus glutamate/malate, paired t test.
4. \(p < 0.001\) versus succinate, \(t\) test; \(p = 0.015\) versus glutamate/malate, \(t\) test.
5. Dixon plot calculated from Fig. 2.

### Figure 2

**Analysis of Zn\(^{2+}\) inhibition of mitochondrial respiration.** Dixon plots of oxygen consumption by mitochondria in the presence of varying concentrations of Zn\(^{2+}\). The value of \(V\) for each point was calculated from the ratio of the experimental respiration rate to the average respiration rate for control samples from the same preparation, which was arbitrarily assigned a value of 1. The different symbols represent four or five independent preparations. A, succinate. B, glutamate/malate. C, \(\alpha\)-KG.

### Figure 3

**Effect of Zn\(^{2+}\) on MDH and GDH activities.** Dixon plots of activities of purified MDH (160 milliunits/ml; A) and GDH (300 milliunits/ml; B) in the presence of varying concentrations of Zn\(^{2+}\).

### References

52. Direct dilution of KGDHC in storage buffer into assays results in concentrations of 2–5 \(\mu\)M for both EDTA and EGTA and 0.1–0.3 \(\mu\)M BSA in the final assay mixture. Therefore, the residual concentration of chelators was high enough to interfere with the determination of inhibition constants for metal ions. EDTA, EGTA, and BSA were simultaneously removed from the enzyme preparation by rapid gel filtration chromatography on a Sephadex 200 column. As expected, the apparent \(K_{\text{app}}\) for Zn\(^{2+}\) decreased after these chelators were removed from the enzyme stock. Denaturing gel electrophoresis stained with Coomassie Blue confirmed that G-150 chromatography removed >97% of the BSA initially found in the enzyme preparation (data not shown). KGDHC subjected to gel filtration was used in all of the experiments presented below.

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The hypothesis was investigated by varying the concentration of Zn\(^{2+}\) genases, in addition to inhibiting electron transport. This hypothesis is supported by the coincidence of elevated intracellular glutamate and reduced carbohydrate flux that was reported in some disease states (16–21) led us to hypothesize that elevated Zn\(^{2+}\) is a target for Zn\(^{2+}\) detoxification (32, 33). However, Zn\(^{2+}\) directly inhibits NADH-producing dehydrogenases (16–21) as indicated. The values for \(K_{\text{app}}\) determined by least square fitting of the linear regression lines for 1, 2, and 4 \(\mu\)g/ml KGDHC are (mean ± S.E.) 0.19 ± 0.08, 0.22 ± 0.07, and 0.34 ± 0.06 \(\mu\)M, respectively. C, inhibition by EDTA. Inhibition by residual divalent ions in the buffer preparation can be prevented by addition of submicromolar EDTA prior to assay. Plot of \(V_{\text{app}}\) versus EDTA concentration. The lines are independent linear regression fits to data points corresponding to 0–0.3 and 0.4–1.0 \(\mu\)M EDTA, respectively. The lines intersect at 0.3 \(\mu\)M Zn\(^{2+}\).

### DISCUSSION

Initial observations that Zn\(^{2+}\)-inhibited mitochondrial respiration (32, 33) led to a series of studies that culminated in the proposal that the bc\(_1\) complex of the electron transport chain is a target for Zn\(^{2+}\) (34, 35). However, Zn\(^{2+}\)-induced inhibition of upstream dehydrogenases was not systematically examined (see the Introduction). The coincidence of elevated intracellular free Zn\(^{2+}\) and reduced carbohydrate flux that was reported in some disease states (16–21) led us to hypothesize that elevated intracellular Zn\(^{2+}\) directly inhibits NADH-producing dehydrogenases, in addition to inhibiting electron transport. This hypothesis was investigated by varying the concentration of Zn\(^{2+}\) within the physiological range (37) while monitoring respiration of isolated mitochondria. The effect of Zn\(^{2+}\) on the activity of selected mitochondrial dehydrogenases was also determined.

The changes in mitochondrial respiration that were induced by Zn\(^{2+}\) are unlikely to be due to changes in mitochondrial integrity. Zn\(^{2+}\) has been implicated in several mitochondrial changes, such as ion transport (55–57) and membrane swelling (33, 56, 58, 59). Membrane swelling has been associated with the opening of the mitochondrial permeability transition pore, loss of membrane potential, and uncoupling of respiration (60). However, Mg\(^{2+}\) was included in the respiration buffer. Control experiments have established that 3 \(\text{mM}\) Mg\(^{2+}\) prevents induction of the permeability transition by Zn\(^{2+}\) (2).

The absence of any absorbance change in mitochondrial suspensions also argues against Zn\(^{2+}\)-induced disruption of mitochondrial membrane integrity.

Zn\(^{2+}\) Inhibits Mitochondrial Respiration Supported by α-KG, Glutamate/Malate, or Succinate—The order of sensitivity to Zn\(^{2+}\) inhibition among the various substrates tested in intact mitochondria was α-KG > glutamate/malate > succinate (Figs. 1 and 2; Table I). Electrons (reducing equivalents) from glutamate/malate or α-KG enter the electron transport chain as NADH at complex I. Electrons from succinate enter the electron transport chain as FADH\(_{2}\) at complex II. Electrons from both complexes I and II then feed into the Q cycle portion of

## Table II

| Series | Preincubation time with Zn\(^{2+}\) (min) | Reaction duration prior to \(V_{\text{app}}\) interval (min) | \(K_{\text{app}}\) (μM ± S.E.) |
|--------|------------------------------------------|-------------------------------------------------|--------------------------|
| Preincubation | 0 | 0 | 0.54 ± 0.03 |
| | 17 | 0 | 0.42 ± 0.04 |
| | 35 | 0 | 0.46 ± 0.07 |
| | 61 | 0 | 0.50 ± 0.10 |
| | 78 | 0 | 0.50 ± 0.06 |
| Postincubation | 0 | 0 | 0.56 ± 0.05 |
| | 35 | 0 | 0.42 ± 0.05 |
| | 61 | 0 | 0.26 ± 0.06 |
| | 78 | 0 | 0.18 ± 0.06 |

* \(p = 0.003\), where \(p\) was calculated for the slope of the regression line of reaction duration interval versus \(K_{\text{app}}\).

## Fig. 4

KGDHC inhibition by Zn\(^{2+}\) and disinhibition by EDTA. Chelator-free KGDHC was prepared and diluted into a standard reaction mixture, as described under "Materials and Methods." A, reaction progress curves for KGDHC in the presence of indicated concentrations of Zn\(^{2+}\) with no preincubation. Concentration of KGDHC was 4 \(\mu\)g/ml (~22 milliunits/ml). B, Dixon plot of Zn\(^{2+}\) inhibition at three input concentrations of KGDHC, as indicated. The values for \(K_{\text{app}}\) determined by least square fitting of the linear regression lines for 1, 2, and 4 \(\mu\)g/ml KGDHC are (mean ± S.E.) 0.19 ± 0.08, 0.22 ± 0.07, and 0.34 ± 0.06 μM, respectively. C, disinhibition by EDTA. Inhibition by residual divalent ions in the buffer preparation can be prevented by addition of submicromolar EDTA prior to assay. Plot of \(V_{\text{app}}\) versus EDTA concentration. The lines are independent linear regression fits to data points corresponding to 0–0.3 and 0.4–1.0 \(\mu\)M EDTA, respectively. The lines intersect at 0.3 \(\mu\)M Zn\(^{2+}\).
complex III (cytochrome bc₁ complex). It was reported that purified cytochrome bc₁ is inhibited by submicromolar concentrations of Zn²⁺ (34, 35). However, the greater Zn²⁺ sensitivity observed for intact mitochondria respiring on complex I substrates (α-KG or glutamate/malate) than on complex II substrate (succinate) (Fig. 2) indicates that a site upstream from Zn²⁺-mediated inhibition, at least for intact mitochondria. The greater sensitivity of α-KG stimulated respiration suggested that KGDHC might be the most Zn²⁺ sensitive upstream factor. We therefore compared the Zn²⁺ sensitivity of KGDHC to other dehydrogenases that oxidize mitochondrial substrates used in this study.

KGDHC Sensitivity to Zn²⁺ Inhibition—The activity of purified KGDHC is potently inhibited by Zn²⁺ (Fig. 4), consistent with the high Zn²⁺ sensitivity of α-KG-stimulated mitochondrial respiration. Purified GDH is ~15-fold less sensitive to Zn²⁺, and Zn²⁺ does not inhibit SDH and MDH activities. The weaker but appreciable inhibition of mitochondrial respiration observed in the presence of substrates other than α-KG suggests that there may be other mitochondrial targets for Zn²⁺. One such possible target is the succinate transporter, which is inhibited by Zn²⁺ in bacteria (61). Also, some inhibition of complex I by Zn²⁺ cannot be excluded by our data.

Zn²⁺ Inhibition of KGDHC Is Time- and Activity-dependent—Zn²⁺ inhibits KGDHC activity with a Kᵦ of ~0.4 μM for the intact, purified enzyme (Fig. 4B) and Kᵦ of ~1 μM for intact mitochondria respiring on α-KG (Fig. 2C). Slow onset of Zn²⁺ inhibition was observed for the isolated enzyme in the presence of Zn²⁺ and α-KG (Fig. 4A). Slow onset and slow reversal (Fig. 5A) of inhibition is often observed with tightly binding inhibitors (54). The values of Kᵦ reported here represent an upper limit for the true Kᵦ because of two considerations. First, Zn²⁺ inhibition increases with time as illustrated in Table II, but the determination of Kᵦ was based on substrate formed during the first 15 min after initiation of the reaction. A second factor that may contribute to an excessively high estimate of the Kᵦ for Zn²⁺ is the presence of ~0.3 μM endogenous Zn²⁺ or other chelatable divalent ion inhibitors within the KGDHC reaction mixture (Fig. 4C). Taken together, these considerations suggest that the actual Kᵦ may be ~0.1 μM or less.

The Kᵦ for KGDHC and Zn²⁺ progressively diminishes with continued substrate cycling in the presence of α-KG. In contrast, preincubation in the absence α-KG has no effect on the value of Kᵦ (Table II). Substrate cycling may be required because Zn²⁺ binds to a site on the enzyme that becomes available only during turnover. For example, Zn²⁺ might inhibit KGDHC activity by binding to the lipoyl prosthetic group of E2. The lipoyl group (i.e. [LipS₂]-E2) is oxidized in the resting state of the enzyme. Therefore, strong (bidentate) binding is only possible after complete reduction of the lipoyl group to a di-thiol (i.e. [Lip(SH)₂]-E2), which requires the presence of substrates (Equations 2 and 3). Similarly, E3 in the resting state contains a disulfide that is reduced to a dithiol by Lip(SH)₂-E2 (62), which is available only during enzyme cycling. If Zn²⁺ inhibition of E3 involves dithiol binding, then it necessarily requires enzyme cycling. Reduced E3 is only likely to exist when [Lip(SH)₂]-E2 is available, which is substrate-dependent. Other explanations for slow onset of inhibition are possible. For example, Zn²⁺ may form an inhibitory complex with one of the enzyme products (e.g. NADH; see below) generated during enzyme cycling. Preliminary experiments of the subunit reactions suggest that both E1/E2 and E3 activities are sensitive to Zn²⁺ inhibition. Further studies are needed to clarify the precise mechanism by which Zn²⁺ inhibits intact KGDHC.

Partial Reversibility of Zn²⁺ Inhibition of KGDHC—The recovery of KGDHC activity is independent of EDTA concentration beyond that which is necessary to stoichiometrically bind Zn²⁺. This finding indicates that dissociation of Zn²⁺ from the inhibited complex is a unimolecular process followed by sequestration of free Zn²⁺ by EDTA. The dependence of recovery of KGDHC activity on both the dose and duration of exposure to Zn²⁺ suggests that initially the inhibited enzyme complex is fully reversible but that it is subsequently converted to a second irreversible or slowly reversible form. In the terminology of Morrison and Walsh (54), the first complex exhibits “slow” inhibition, whereas the second is characteristic of “slow-tight” inhibition. The gradual onset of Zn²⁺ inhibition and gradual recovery after addition of EDTA is characteristic of a slow binding complex. The development of stable inhibition characterized by a gradual loss of EDTA reversibility reflects the conversion from a slow to a slow-tight binding complex. The gradual development of inhibition is not simply a consequence of substrate consumption, because the fraction of substrate consumed is 5% or less.

Physiological Implications—Our data demonstrate that concentrations of Zn²⁺ reported to occur in cultured hepatocytes (37) inhibit KGDHC activity. KGDHC activity within intact liver mitochondria is partly inhibited by 0.4 μM and completely inhibited by 3.2 μM Zn²⁺ (Fig. 1); an even lower range of Zn²⁺ concentrations (Fig. 4) inhibits purified KGDHC. The estimated range of cytosolic Zn²⁺ concentration in cultured hepatocytes (0.6–2.7 μM) (37) overlaps with the range of mitochondrial sensitivity observed in this report. The correspondence between the availability in cells and the sensitivity of mitochondria leads us to propose that modest fluctuations of intracellular Zn²⁺ may play a physiological role in the regulation of mitochondrial energy metabolism. Inhibition of KGDHC by chronic low doses of Zn²⁺ or high doses of short duration is readily reversible (Fig. 5). Therefore, mild or transient elevation of cellular free Zn²⁺ levels would be predicted to transiently reduce KGDHC-dependent respiration. More severe or prolonged exposure to elevated Zn²⁺ would result in irreversible inactivation of some or all of the available KGDHC and could lead to persistent inhibition of mitochondrial respiration. Preliminary experiments indicate that the mitochondrial pyruvate dehydrogenase complex can also be inhibited by micromolar concentrations of Zn²⁺. More precise definition of the potential role of Zn²⁺ as a physiological regulator of mitochondrial oxidative metabolism will require further studies.

Pathological Implications—Changes in the distribution of free Zn²⁺ have been described for several disorders, including hypoxia/ischemia (2, 3) and Alzheimer’s disease (63–65). Zn²⁺ influx into cells is associated with neuronal death (2, 3, 10). Moreover, direct involvement of Zn²⁺ in cytotoxic mitochondrial changes, including free radical formation, have been suggested (7, 8, 66).

Oxidative energy metabolism is impaired in many neurodegenerative disorders (22–24). In particular, KGDHC activity in brain is reduced in Alzheimer’s disease and a number of other neurodegenerative disorders (25, 26, 29, 67), but the possible relationship of the enzyme deficiency to Zn²⁺ levels in these conditions has not yet been explored. The decrease in KGDHC activity in Alzheimer’s disease brain has been reported to exceed the decrease in KGDHC protein (67). Imbalances in the distribution of Zn²⁺ (63–65) in Alzheimer’s disease brain may contribute to the loss of KGDHC activity.

The data in the present study indicate that damage because of pathological elevations of Zn²⁺ may occur, at least in part, through inhibition of α-KG oxidation at the KGDHC-catalyzed step. As discussed above, shorter exposure to relatively lower
levels of Zn\(^{2+}\) is associated with reversible inhibition of KGDHC, and low levels of Zn\(^{2+}\) may thus play a role in metabolic regulation. However, prolonged exposure to higher concentrations of Zn\(^{2+}\) may lead to slow and incomplete recovery from inhibition of KGDHC activity and mitochondrial oxidative function (Fig. 5). The present findings raise the possibility that extreme elevations of Zn\(^{2+}\) that may exist under pathological conditions lead to such severe and long-lasting inhibition of α-KG oxidation in mitochondria that they contribute to cell death. This possibility also needs to be tested by further experiments.

**Conclusion—**The data reported here raise the possibility of a previously unsuspected role of Zn\(^{2+}\) in the normal regulation of metabolism, namely, at the KGDHC-catalyzed step of mitochondrial oxidative disorders and heart disease, particularly at the step catalyzed by KGDHC. The current studies indicate that Zn\(^{2+}\) may well play a critical role in these disorders. Further studies of the role of Zn\(^{2+}\) in the regulation of energy metabolism in health and disease are warranted.

**REFERENCES**

1. Frederickson, C. J. (1989) *Int. Rev. Neurobiol.* 31, 145–238
2. Tonder, N., Johansen, F. F., Frederickson, C. J., Zimmer, J., and Diemer, N. H. (1988) *Brain Res.* 460, 317–321
3. Cunnane, S. C. (1988) *Zinc: Clinical and Biochemical Significance* (Tanzi, R. E., and Wasco, M. D., eds) pp. 91–102, Humana Press, New York
4. Frederickson, C. J., Hernadez, M. D., and McGinty, J. F. (1989) *Science* 247, 245–247
5. Link, T. A., and von Jagow, G. (1995) *J. Biol. Chem.* 270, 25001–25006
6. Kita, K., Konishi, K., and Anraku, Y. (1984) *J. Biol. Chem.* 259, 3368–3374
7. Cleckner, J. W., and Brand, I. A. (1973) *J. Pharmacol. Toxic. Methods* 38, 1–6
8. Berg, A., and Besnard, A. M. (1990) *Arch. Biochem. Biophys.* 272, 297–304
9. Masuoka, J., Hegenauer, J., Van Dyke, B. R., and Saltman, P. (1993) *J. Neurochem.* 61, 577–584
10. Koh, J. Y., and Choi, D. W. (1994) *J. Pharmcol. Toxic. Methods* 31, 101–102
11. Weiss, J., Hartley, D., Koh, J., and Choi, D. W. (1993) *Ann. Neurol.* 33, 39–47
Zn$^{2+}$ Inhibits $\alpha$-Ketoglutarate-stimulated Mitochondrial Respiration and the Isolated $\alpha$-Ketoglutarate Dehydrogenase Complex

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J. Biol. Chem. 2000, 275:13441-13447.
doi: 10.1074/jbc.275.18.13441

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