Zinc Irreversibly Damages Major Enzymes of Energy Production and Antioxidant Defense Prior to Mitochondrial Permeability Transition*

Received for publication, December 12, 2006, and in revised form, May 17, 2007 Published, JBC Papers in Press, June 12, 2007, DOI 10.1074/jbc.M611376200

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Recent observations point to the role played by Zn\(^{2+}\) as an inducer of neuronal death. Two Zn\(^{2+}\) targets have been identified that result in inhibition of mitochondrial respiration: the bc\(_1\) center and, more recently, f1 center (13, 14). More recently, we demonstrated that Zn\(^{2+}\) is also a mediator of oxidative stress, leading to mitochondrial failure, release of apoptotic peptides, and neuronal death.

We now present evidence, by means of direct biochemical assays, that Zn\(^{2+}\) is imported through the Ca\(^{2+}\) uniporter and directly targets major enzymes of energy production (lipoamide dehydrogenase) and antioxidant defense (thioredoxin reductase and glutathione reductase). We demonstrate the following. (a) These matrix enzymes are rapidly inhibited by application of Zn\(^{2+}\) to intact mitochondria. (b) Delayed treatment with membrane-impermeable chelators has no effect, indicating rapid transport of biologically relevant quantities of Zn\(^{2+}\) into the matrix. (c) Membrane-permeable chelators stop but do not reverse enzyme inactivation. (d) Enzyme inhibition is rapid and irreversible and precedes the major changes associated with the mitochondrial permeability transition (MPT). (e) The extent and rate of enzyme inactivation linearly correlates with the MPT onset and propagation. (f) The Ca\(^{2+}\) uniporter blocker, Ruthenium Red, protects enzyme activities and delays pore opening up to 2 μM Zn\(^{2+}\). An additional, unidentified import route functions at higher Zn\(^{2+}\) concentrations. (g) No enzyme inactivation is observed for Ca\(^{2+}\)-induced MPT. These observations strongly suggest that, unlike Ca\(^{2+}\), exogenous Zn\(^{2+}\) interferes with mitochondrial NADH production and directly alters redox protection in the matrix, contributing to mitochondrial dysfunction. Inactivation of these enzymes by Zn\(^{2+}\) is irreversible, and thus only their de novo synthesis can restore function, which may underlie persistent loss of oxidative carbohydrate metabolism following transient ischemia.

Recently a growing number of reports have linked changes in intracellular free Zn\(^{2+}\) to pathological processes, particularly in the nervous system (1). Elevated Zn\(^{2+}\) has been implicated in neuronal death following ischemia (2) and excitotoxicity (3). The role of Zn\(^{2+}\) in Alzheimer disease has been controversial (4), although a recent study strongly associates elevated cortical Zn\(^{2+}\) with Alzheimer disease diagnosis and severity of dementia (5).

One mechanistic theme that has emerged is that elevated intracellular Zn\(^{2+}\) is correlated with mitochondrial dysfunction, loss of mitochondrial defenses, and increased production of reactive oxygen species (1, 6–10). Although Ca\(^{2+}\) is a recognized physiological inducer of mitochondrial permeability transition (MPT) pore, the role of Zn\(^{2+}\) in mitochondria dysfunction is a newly arising question. Zn\(^{2+}\) is a much more potent inducer of MPT than Ca\(^{2+}\) (i.e. it works at doses at least an order of magnitude lower than those needed for Ca\(^{2+}\) (9, 11)). Early publications demonstrated Zn\(^{2+}\) inhibition of mitochondrial respiration (12), which was attributed to the cytochrome bc\(_1\) center (13, 14). More recently, we demonstrated that Zn\(^{2+}\) inhibits mitochondrial matrix multienzyme complexes (15, 16). Other targets have been identified (11, 17), including pore-forming proteins in the outer membrane (18). There is also evidence for Zn\(^{2+}\) import through the calcium uniporter (11, 19, 20), and possibly, an additional unknown import route (20).

Elucidation of plausible targets for Zn\(^{2+}\) is complicated by the fact that the mechanistic details underlying the classical phenomenon of Ca\(^{2+}\)-induced MPT itself are unclear. The composition of the pore complex is still disputed (21, 22). In this work, we addressed the question of Zn\(^{2+}\) targets in MPT using a quantitative approach that is well established for the study of enzyme mechanism. We employed analysis of the combined effects of Ca\(^{2+}\) and Zn\(^{2+}\) on the time course of mitochondrial swelling, whereas varying the concentrations of both metals. This “enzyme kinetics” approach has been complemented by the direct assay of enzymatic activities of flavin-dependent thiol reductases in the mitochondrial matrix during the course of metal-induced swelling. Kinetic analysis provides evidence for both independent and competing sites of action for Ca\(^{2+}\) and Zn\(^{2+}\) that contribute to MPT induction. We also present direct evidence for the impairment of these major enzymes of energy production and antioxidant defense in mitochondrial

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*This work was supported by National Institutes of Health NINDS Grant NS38741 (to A. M. B.). 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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‡‡ The abbreviations used are: MPT, mitochondrial permeability transition; DTNB, dithio-nitrobenzoic acid; GR, glutathione reductase; TR, thioredoxin reductase; GR + TR, combined glutathione and thioredoxin reductases; LA, lipoamide; LADH, LA dehydrogenase; TPEN, N,N,N’,N’-tetrakis (2-pyridylmethyl)ethylenediamine.
matrix in the course of Zn$^{2+}$-induced pore opening, which is
distinct from the mechanism of Ca$^{2+}$-induced pore opening.

**EXPERIMENTAL PROCEDURES**

Reagents—NADH, NADPH, α-lipoamide (n-6,8-thiocic acid
amide), oxidized glutathione, dithio-nitrobenzoic acid
( DTNB), recombinant Escherichia coli thioredoxin, Tris,
HEPES, ZnCl$_2$, EDTA, N,N,N',N'-tetrakis(2-pyridylmethyl)
ethylenediamine (TPEN), Ruthenium Red, alamethicin, suc-
cinic acid, rotenone, glutamic acid, malic acid, isocitric acid,
α-ketoglutaric acid, and KOH were from Sigma. All reagents
were “SigmaUltra” grade, if available, to reduce the possibility
of contamination by divalent cations. Me$_2$SO was from Fisher
Scientific, and HCl was from J. T. Baker, Inc. All solutions were
prepared using distilled, deionized water with >16 megao-
hms/cm of resistance.

NADPH and NADH were freshly prepared as 10 mM stock
solutions in water. Oxidized glutathione and DTNB were
freshly prepared as 30 and 60 mM stock solutions in water and
ethanol, respectively. Lipoamide (LA) was dissolved in Me$_2$SO
(25 mM) and was used to make 0.2–2 mM LA solutions in 50 mM
Tris-HCl buffer, pH 7.5. ZnCl$_2$ was made as a 10 mM stock
solution in water.

**Mitochondria Preparation and MPT Studies**—Rat liver
mitochondria were isolated as described earlier (23). Mito-
chondrial suspensions (50–60 mg of protein/ml) were kept
on ice for no longer than 6 h prior to MPT and/or enzymatic
activity measurements.

MPT experiments were performed with 2 mg/ml mitochon-
dria suspended in 0.25 M sucrose, 5 mM potassium HEPES, pH
7.4, and 5 mM potassium succinate as a respiration substrate,
unless otherwise stated. 100-μl aliquots were placed into a
microplate, and the changes in absorbance at 540 nm were
recorded using a plate reader, as described below. The temper-
ature was maintained at 26 °C. Concentrations of Ca$^{2+}$ and
Zn$^{2+}$ were varied within the range of 1–64 and 0.3–9 μM,
respectively.

Enzyme activities were measured in parallel with absorbance
monitoring. At varied intervals, aliquots were removed, and
enzyme activities were measured as described below. In exper-
iments with TPEN and EDTA chase, mitochondria were loaded
with Zn$^{2+}$, and then 1-ml aliquots were transferred into wells
containing EDTA or TPEN after 1-, 2-, 3-, and 4-min incuba-
tions. In experiments with Ruthenium Red, it was added to
mitochondria simultaneously with Zn$^{2+}$ loading.

**Enzymatic Assays**—All enzymatic assays were performed in
a 96-well plate reader (SpectraMax Plus, GE Healthcare) with a
200-μl reaction mixture per well at 20 °C. The absorbance of
DTNB (ε$_{412}$ = 13.6 mM$^{-1}$ cm$^{-1}$ (24)) was monitored. The light
path was 0.43 cm. All reactions were run in 0.2 M Tris-HCl, pH
7.4, containing 5 mM EDTA and either 0.1 mg/ml alamethicin
or 0.1% Nonidet P-40 to permeabilize mitochondria. (No dif-
fERENCE in recovered activity was observed between alamethicin
and Nonidet P-40 treatments.) GR + TR activity was measured
with 0.2 mM NADPH, 2 mM DTNB, and 0.1 mM oxidized glu-
thathione. LADH activity was measured in a mixture containing
0.2 mM NADH, 2 mM DTNB, and 0.1 mM lipoamide. Reactions
were initiated by the addition of mitochondrial aliquots (10 μl
for LADH activity and 50 μl for TR + GR activity) to the reac-
tion buffers. Preincubation with EDTA in the presence of ala-
methicin did not result in higher activity. We note that DTNB-
based detection has a significant advantage over monitoring
disappearance of NAD(P)H at 340 nm because it provides a
4-fold increase in the absorbance change per unit reaction. In
addition, we observed that the use of DTNB as a terminal elec-
tron acceptor rescues LADH from product inhibition, i.e.
removes reduced lipoamide from the reaction mixture. Dis-
CRImination between the enzymes took advantage of differ-
ences in catalytic properties of the enzymes, i.e. the absence
of LADH activity with NADPH, with DTNB alone; the absence
of TR and GR activity with NADH; and reduced GR activity in
the absence of oxidized glutathione. In all cases, a linear slope was
observed for a minimum of 20 min.

**RESULTS**

Description of Microplate-based Ca$^{2+}$ and Zn$^{2+}$ Swelling
Titrations—The MPT is commonly monitored as changes in
light scattering, which is especially pronounced for liver mito-
chondria. It is well established that changes in mitochondrial
light scattering correspond to matrix swelling associated with
pore opening (25). Swelling can conveniently be recorded spec-
trophotometrically as a decrease in the apparent absorbance at
540 nm (i.e. increased transmittance). Our laboratory intro-
duced the use of a microtiter plate reader to collect experimen-
tal data on mitochondria swelling (23, 26). The advantage of
this approach is that it allows many different experimental con-
ditions to be monitored simultaneously and directly compared.
Henceforth, we will refer to absorbance changes as the primary
output signal corresponding to MPT-associated mitochondrial
swelling.

An example of a two-dimensional titration is shown in Fig.
1A, where two different MPT effectors are titrated simulta-
aneously. In Fig. 1A, the far left column presents the titration
against increasing concentrations of Ca$^{2+}$ in the absence of
added Zn$^{2+}$, whereas the other columns contain different fixed
concentrations of Zn$^{2+}$. Conversely, the highlighted row pre-
sents the titration against Zn$^{2+}$ in the absence of Ca$^{2+}$, whereas
the other rows contain either EDTA or a different fixed concen-
tration of Ca$^{2+}$. This experimental format allows simultaneous
generation of a complete set of titration data for two effectors.
Quantitative relationships between these effectors can there-
fore be determined from analysis of a single experimental run,
eliminating the confounding effects of variations in reagent
concentration or aging of the mitochondria.

The data presented in Fig. 1B (Zn$^{2+}$ only) and Fig. 1C (Ca$^{2+}$
only) appear superficially similar and might lead one to con-
clude (erroneously) that Zn$^{2+}$ and Ca$^{2+}$ are qualitatively simi-
lar, except that Zn$^{2+}$ is ~20-fold more potent Ca$^{2+}$. It is true
that increasing concentrations of either Ca$^{2+}$ or Zn$^{2+}$ acceler-
ate pore opening by shortening the lag period, and both agents
increase the maximum rate of swelling. However, this first
impression is incomplete, as we will show below.

**Kinetic Model of Swelling Curves**—The sigmoidal shape of
the swelling time course is characterized by the lag period and
maximum slope (Fig. 1, inset). The swelling time course
resembles the shape of a sigmoid (Fig. 1, inset), particularly
for faster transitions. The simplest reaction model (27) for sigmoidal kinetics is a series of two consecutive steps for mitochondria transformation via at least one intermediate, which has the same absorbance characteristics as the original state. In this model, only the final state manifests itself by the changes in absorbance. This reaction model is described in Scheme 1.

$$k_1 + k_{	ext{Ca}^{2+}}$$

M → M_{\text{intermediate}} → M_{\text{swollen}} + Ca^{2+}

SCHEME 1

The first step corresponds to the binding or uptake of Ca$^{2+}$ by mitochondria, presumably by the Ca$^{2+}$ uniporter, and may be reversible. The second step corresponds to the rate-limiting step in the unknown intermediate transformation(s) that eventually lead(s) to pore opening. Similarly, a recent study proposes that MPT is at least a two-step mechanism culminating in pore opening (28).

The effector (e.g. Ca$^{2+}$ or Zn$^{2+}$) may alter any or all of these characteristics by influencing different steps in the transformation chain presented in Scheme 1. The lag period may correspond principally to a “pore-forming event” (28) or a “cristae remodeling event” (29). In contrast, the maximum rate of swelling will reflect contributions from both the processes leading to the initial pore-opening event and the propagation of pore opening through the mitochondrial population. This is because pore opening results in Ca$^{2+}$ release, which increases the concentration of effector in the first step of Scheme 1.

Large-amplitude swelling is a consequence of the pore-opening event that is conjectured to be “all-or-none” in nature (30), i.e. the pore is either open or closed. Once the pore opens, the released calcium is consumed by the remaining coupled mitochondria, leading to progressively greater Ca$^{2+}$ overload to the coupled fraction of mitochondria. Moreover, swollen mitochondria are known to release more calcium than was added initially, further contributing to the overall Ca$^{2+}$ load. In the terminology of chemical kinetics, the Ca$^{2+}$-induced swelling reaction is autocatalytic or product-catalyzed. No analytical solution exists for the complete system of equations describing autocatalytic reactions in chemical kinetics. Nevertheless, kinetic analysis can be applied to separate the “events” represented by different characteristics of the swelling curves.

Analysis of Maximum Rates of Swelling, Mixed Competition of Zn$^{2+}$ and Ca$^{2+}$ Sites in MPT—The maximum rate of swelling, measured as the maximal rate of change of mitochondrial absorbance, has been recognized as an integral characteristic of the process and analyzed in double-reciprocal plots (31). This approach is based on the assumption that the maximum rate corresponds to the establishment of a pseudo-steady state where the concentration of M_{intermediate} reaches the maximum, and for a short time period, its concentration can be considered constant. During this interval, the maximum rate corresponds to the maximum concentration of M_{intermediate} multiplied by the rate constant for its final conversion ($k_2$).

In the most general case, the maximum concentration of M_{intermediate} will be a function of both rate constants (formation and consumption) in Scheme 1. Therefore, if an inducer (such as Zn$^{2+}$) influences both rate constants, the maximum rate may be a complex function of the inducer concentration. Nevertheless, double reciprocal treatment may point to the relationships between two co-varied inducers.

Fig. 2 presents double-reciprocal plots for the maximum rate of swelling. The plots indicate that both Zn$^{2+}$ and Ca$^{2+}$ influence the maximum rate (intercept on the y axis) and apparent “binding constant” (intercept on the x axis) for the site(s) that regulate the swelling process. Stated in other words, the two metals compete in stimulating the swelling process but do not fully substitute for each other. The activation constants for the competitive Zn$^{2+}$ and Ca$^{2+}$ site, determined from the intersection points, are equal to 0.25 and 5 μM, respectively. This corresponds to the 20-fold more potent effect of Zn$^{2+}$ when com-

![Image](image-url)
pared with Ca\textsuperscript{2+} in MPT induction seen by inspection of Fig. 1, B and C.

It is well established that MPT induction involves the Ca\textsuperscript{2+} uniporter (32, 33). As we will show later, Zn\textsuperscript{2+} also enters the mitochondrial matrix through the Ca\textsuperscript{2+} uniporter. The possible identity of the competitive Zn\textsuperscript{2+} and Ca\textsuperscript{2+} site will be commented on in the “Discussion.”

**Analysis of Swelling at Saturating Concentrations, Identification of Kinetically Independent Zn\textsuperscript{2+} and Ca\textsuperscript{2+} Sites**—An enzyme kinetics approach that can help to untangle the relationship between two activators or inhibitors is to study the effect of varying one in the presence of the saturating concentration of the other. If the effectors (i.e., Ca\textsuperscript{2+} and Zn\textsuperscript{2+}) compete for a single “physical site” (which in the case of MPT may be the Ca\textsuperscript{2+} uniporter, a component of the permeability pore, or an unknown matrix enzyme/protein), this site can be completely occupied by saturating concentrations of either metal. No effect will be observed upon the addition of the second effector if the concentration of the first one was saturating. In contrast, if the effector sites are independent, the addition of the second effector at the saturating concentration of the first one will still result in a pronounced change on the time course curve.

In the case of MPT, the concentration of the effector is considered saturating if its subsequent addition causes no major changes in the time course of swelling. In the case of Ca\textsuperscript{2+}, the saturated concentration range starts at \(~30 \mu M\), whereas for Zn\textsuperscript{2+}, it is above \(3 \mu M\).

The existence of two distinct sites in MPT for Ca\textsuperscript{2+} and Zn\textsuperscript{2+} was visualized by the following approach. Varying Zn\textsuperscript{2+} in the presence of saturating Ca\textsuperscript{2+} (Fig. 3A) results in lag period shortening with the maximum slope lines that are nearly parallel. In contrast, varying Ca\textsuperscript{2+} in the presence of saturating Zn\textsuperscript{2+} (Fig. 3B) results in the increase of the maximum slope with no changes in the lag period duration (see the slope lines intersecting at the same time point). This qualitative difference in the concentration-dependent behavior is evidence for the presence of distinct, kinetically independent effector sites Ca\textsuperscript{2+} and Zn\textsuperscript{2+} in MPT. We interpret this qualitative difference in the
following manner. Zn\(^{2+}\) speeds up step(s) that precede large-amplitude swelling with a resulting shortening of the lag period. On the other hand, Ca\(^{2+}\) speeds up propagation of the pore-opening event through the mitochondrial population, resulting in a faster swelling rate.

Irreversible Inactivation of LADH and GR + TR Pools in Zn\(^{2+}\), but not Ca\(^{2+}\), MPT—An important goal for understanding the nature of the Zn\(^{2+}\)-induced shortening of lag is the identification of molecular targets that are altered by Zn\(^{2+}\). We have previously observed that LADH is a target of Zn\(^{2+}\) (16), an NADH-dependent thiol reductase. Therefore, we investigated the impact of Zn\(^{2+}\) treatment upon NAD(P)H-dependent thiol reductase enzyme activities inside mitochondria, whereas simultaneously monitoring the time course of MPT pore opening.

We monitored the activity changes of mitochondrial LADH and GR + TR activities in the course of Zn\(^{2+}\) - and Ca\(^{2+}\)-induced MPT. This was accomplished by exposing coupled mitochondria to Ca\(^{2+}\) or Zn\(^{2+}\) and then assaying residual enzyme activities at different times after the addition of effector. Rapid assay of these matrix enzymes was accomplished by adding mitochondrial aliquots directly to assay mixtures that included detergent or alamethicin, a pore-forming antibiotic peptide, to rapidly expose the matrix contents to enzyme substrates. The assay mixture also included a large excess of EDTA (0.5 mM) to chelate all free Zn\(^{2+}\) or Ca\(^{2+}\) and prevent inhibition by Zn\(^{2+}\) or Ca\(^{2+}\) that had not entered into the mitochondria prior to lysis. Because this procedure also relieved any reversible metal-dependent enzyme inhibition, the changes detected represent irreversible enzyme inactivation. Previous studies demonstrated that reversal of Zn\(^{2+}\) inhibition of 2-oxoglutarate dehydrogenase complex is gradual, as demonstrated by upward curvature of the reaction curves (15). Delayed recovery of activity has been avoided in the current experiments by the use of higher EDTA concentrations (0.5 mM versus 10 \(\mu\)M). The resulting activity measurements show no curvature (see “Experimental Procedures”), in contrast to our previous observations.

Inactivation of LADH (Fig. 4A) and GR + TR (Fig. 4B) occurs largely prior to pore opening (Fig. 4C) and is proportional to Zn\(^{2+}\) concentration. No consistent drop in these enzyme activities was observed (Fig. 4D) upon the addition of sufficient Ca\(^{2+}\) to induce MPT in a similar time scale. Furthermore, inactivation of these enzymes requires that Zn\(^{2+}\) treatment be applied to intact, coupled mitochondria. Fig. 4E illustrates that even high doses of Zn\(^{2+}\) applied to permeabilized mitochondria result in only modest enzyme inhibition when compared with low doses applied to intact mitochondria (Fig. 4F). Taken together, these results suggest that Zn\(^{2+}\) enters the mitochondrial matrix prior to pore opening, resulting in irreversible inactivation of LADH and GR + TR. Enzyme inactivation was observed in coupled mitochondria for all respiration substrates tested (succinate, succinate/rotenone, glutamate/malate, \(\alpha\)-ketoglutarate, isocitrate).
LADH inactivation is well fit by a single exponential (Fig. 4A), whereas GR + TR inactivation is biphasic (Fig. 4B). The initial phase of the GR + TR pool inactivates more rapidly and is more sensitive to Zn$^{2+}$ than LADH, although the residual GR + TR activity remains higher than LADH (Fig. 4F, compare curves). The biphasic response reflects the complex composition of the GR + TR pool. The order of sensitivity to Zn$^{2+}$ is TR $\gg$ LADH $>$ GR.\(^3\) Therefore, we suggest that Zn$^{2+}$ rapidly inactivates TR, whereas GR is less sensitive to Zn$^{2+}$ than LADH.

**TPEN, but Not EDTA, Rescues the Intramitochondrial Enzymes from Zn$^{2+}$**—The enzyme inactivation observed after the addition of Zn$^{2+}$ to intact mitochondria might be due to direct or indirect effects of Zn$^{2+}$.$^1$ An example of the indirect effects of Zn$^{2+}$ is inhibition of the electron transport chain (12–14), which would increase matrix NADH levels and result in over-reduction of LADH. EDTA was previously shown to reverse inhibition of mitochondrial respiration resulting from Zn$^{2+}$ binding to Complex III (17). However, EDTA has almost no effect on the enzyme inactivation time course (Fig. 5, A and B). This suggests that the Zn$^{2+}$ binding site responsible for inactivating thiol enzymes is not on the mitochondrial membrane.

Direct inhibition of matrix enzymes requires Zn$^{2+}$ entry into the matrix. If this is the cause of inactivation of the enzymatic activities, then the enzymes should be rescued from inactiva-

\(^3\) I. G. Gazaryan, I. P. Krasinskaya, S. V. Kazakov, I. V. Uporov, A. B. Gorovits, A. A. Turanov, V. N. Gladyshev, and A. M. Brown, submitted for publication.
Zn\(^{2+}\) Inactivates Intramitochondrial Dithiol Oxidoreductases

more susceptible to the sequence of events, such as oxidative damage, resulting in pore opening. Alternatively, inactivation of flavin-dependent thiol-disulfide oxidoreductases is a marker of Zn\(^{2+}\) penetration into the mitochondrial matrix. Once Zn\(^{2+}\) has entered the matrix, it may interact with other targets that are directly relevant to the mechanism of pore opening.

Conclusions—We have shown that Zn\(^{2+}\) can be imported through the Ca\(^{2+}\) uniporter into the mitochondrial matrix where it inactivates LADH, GR, and TR. Inactivation of LADH will prevent NADH (energy) production because it is the terminal component of the pyruvate, ketogenic, and branched chained dehydrogenase complexes. Antioxidant defense will be severely affected because LADH, TR, and GR are the major generators of reduced thiols in the matrix.

Inhibition of LADH may be the mechanism underlying the loss of \(\alpha\)-ketoglutarate-stimulated respiration in the presence of Zn\(^{2+}\) that we previously reported (15). This is supported by our preliminary observation that matrix \(\alpha\)-ketoglutarate dehydrogenase complex activity is inhibited following treatment of intact mitochondria with Zn\(^{2+}\), with kinetics that appear to parallel the inactivation of LADH (data not shown). Zn\(^{2+}\) is mobilized following brain ischemia/reperfusion. Therefore, Zn\(^{2+}\) inhibition of LADH may underlie the persistent loss of flux through pyruvate dehydrogenase complex observed after transient ischemia in either heart or brain (35, 36). Since pyruvate dehydrogenase complex is the entry point for carbohydrate oxidative metabolism into the Krebs cycle, Zn\(^{2+}\) inhibition may be the cause of the well documented depression of post-infarct oxygen utilization (37).

In addition to the inactivation of LADH, Zn\(^{2+}\)-induced irreversible inactivation of glutathione reductase and thioredoxin reductase will likely contribute to gradual oxidation of matrix thiols, consistent with the data that the drop in enzyme activities parallels the delay in MPT induction. This raises the question of whether the thioredoxin reductase/thioredoxin system is directly involved in controlling the thiol ("S") site in MPT (38). Future work will be directed toward the elucidation of a molecular link between the inactivation of these important enzymes by Zn\(^{2+}\) and the opening of MPT.

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