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News & Views

What is the Functional Significance of the Unique Location of Glutaredoxin 1 (GRx1) in the Intermembrane Space of Mitochondria?

HARISH V. PAI,[1] DAVID W. STARKE,[1] EDWARD J. LESNEFSKY,[2,3] CHARLES L. HOPPEL,[1,2] and JOHN J. MIEYAL[1,3]

ABSTRACT

Glutaredoxins (GRx) catalyze reversible protein glutathionylation. They are implicated in sulfhydryl homeostasis and regulation of redox signal transduction, controlling various cellular processes like DNA synthesis, defense against oxidative stress, apoptosis signaling, and DNA-binding of transcription factors. Two isoforms of GRx are well characterized in mammals: GRx1, the "cytosolic" form, and GRx2, the "mitochondrial" form. Here we report documentation of GRx1 in mitochondria, localized exclusively in the intermembrane space and segregated from GRx2, localized exclusively in the mitochondrial matrix. We hypothesize that GRx1 and GRx2 in their unique locations regulate different functions of the mitochondria *via* reversible *S*-glutathionylation. *Antioxid. Redox Signal.* 9, 2027–2033.

CELLULAR FUNCTIONS OF GLUTAREDOXIN

The redox status of sulfhydryl groups is important to cellular functions such as the synthesis and folding of proteins and the regulation of the structure and activity of enzymes, receptors, and transcription factors. To maintain the cellular thiol status of the cysteine residues of proteins under conditions of redox flux, living cells possess two major thiol-disulfide oxidoreductase systems, the thioredoxin (TRx)/thioredoxin reductase system and the glutaredoxin (GRx)/glutathione (GSH)/GSSG reductase system, both of which use reducing equivalents from NADPH. GRx, also known as thioltransferase, was first discovered as a catalyst for GSH-dependent reduction of ribonucleotide reductase in *Escherichia coli* mutants lacking TRx (14). Subsequently, various forms of GRx have been characterized in bacteria, fungi, plants, and animals, including humans, and these GRx enzymes have been reported to catalyze a variety of apparently divergent reactions, including reduction of arsenate and dehydroascorbate (9, 26). However, mammalian GRx enzymes are most specific as efficient catalysts for reduction of glutathione-containing mixed disulfides, in particular protein-cys-S-S-glutathione (*i.e.,* deglutathionylation of protein-SSG) (13, 32). GRx1 is the best-studied and most thoroughly characterized mammalian deglutathionylating enzyme, exhibiting rate enhancements on the order of 10^4 for protein-SSG substrates (6) and representing the majority of intracellular deglutathionylase activity (6). It has been reported to catalyze deglutathionylation of many different protein substrates *in vitro* and *in situ*, and it is far more efficient than other thiol-disulfide oxidoreductase enzymes in catalyzing protein deglutathionylation *in vitro* (*e.g.,* GRx1 displays a 5,000-fold greater k_{cat}/K_M for Cys-SSG as substrate *vs.* thioredoxin) (6). The deglutathionylating activity of GRx1 has been implicated in the regulation of key physiologic and signaling events, including actin polymerization, vasodilation, cellular hypertrophy, transcription factor activation, and propagation of apoptosis (32).

Departments of [1]Pharmacology and [2]Medicine, Case Western Reserve University, and [3]Louis Stokes Veterans Administration Medical Center, Cleveland, Ohio.

DIFFERENT ISOFORMS OF MAMMALIAN GLUTAREDOXIN

Until recently, evidence existed for only a single genetic form of GRx in mammals, corresponding to the cytosolic protein, now known as GRx1. Based on sequences corresponding to the GRx1 active-site motif (CXXC) and the glutathionyl stabilization site, a second form of mammalian glutaredoxin (GRx2) was found with N- and C-terminal leader sequences that would target it to mitochondria, and possibly to nuclei (11, 25). GRx2 is <35% identical to GRx1, and their polyclonal antibodies do not crossreact. Nevertheless, GRx2 does possess specific catalytic deglutathionylase activity like GRx1 (17), and it operates *via* a double-displacement catalytic mechanism like GRx1 (12). An inactive dimeric form of GRx2, in complex with an iron–sulfur cluster, has been characterized, and it may represent the steady-state form of the enzyme (3, 16, 22).

Nevertheless, a number of studies have implicated GRx2, presumably localized in the mitochondrial matrix, as playing a functional role in redox homeostasis of the mitochondria (2, 8, 23). In particular, GRx2 was reported potentially to regulate the redox status of complex I *via* reversible *S*-glutathionylation (2). Overexpression studies in HeLa cells and human lens epithelial (HLE-B3) cells suggested a role for GRx2 in the protection of mitochondrial integrity, specifically in preventing cardiolipin release and disruption of the mitochondrial permeability transition ($\Delta\psi_m$) after treatment with exogenous oxidants (8). Moreover, a broader function for GRx2 in general cytoprotection was suggested by observations that knockdown of GRx2 sensitized HeLa cells to death induced by phenylarsine oxide and doxorubicin, agents that promote oxidative stress (23).

Contemporary with the discovery of the mammalian GRx2, a number of other studies documented glutaredoxin activity and particularly GRx1 content in mitochondria of various tissues (7, 18, 28) and implicated GRx1 in reactivation of the mitochondrial enzyme αKGDH *in vitro* (29). In addition, Northern blot analyses for GRx1 and activity assays implicated GRx1 in regulation of complex I activity *in vivo* (18). The observations by us and others indicating a presence and functional impact of GRx1, the "cytosolic form," in mitochondria prompted us to investigate the specific localization of GRx1 and GRx2 in mitochondria. By analogy to Cu,Zn-SOD, the "cytosolic form" of SOD, which is documented to be present also in the mitochondrial intermembrane space (IMS) (30), we hypothesized that GRx1 would be localized in the IMS of mitochondria, where mediators of apoptosis are localized.

t-BID AND PERMEABILIZATION OF THE OUTER MITOCHONDRIAL MEMBRANE

The prominent role of mitochondria in mediating cell death is well established in a variety of cellular systems. Involvement of mitochondria in apoptosis is regulated by proteins of the Bcl-2 (B-cell lymphatic–leukemia protooncogene 2) family, which act biochemically by altering the properties of mitochondrial membranes to facilitate the release of apoptogenic proteins like cytochrome *c*, which activate the caspase cascade of cell degradation. In particular, Bid (BH3-interacting domain) death agonist belongs to the Bcl-2 protein family. Cleavage of Bid by caspase-8 generates 15-kDa truncated Bid (t-Bid), which is a much more potent death agonist than full-length Bid. Many studies have documented that t-Bid permeabilizes specifically the outer mitochondrial membrane (OMM), releasing intermembrane space proteins, including the well-known markers adenylate kinase 2 (AK-2) and cytochrome *c* (cyt c) (*e.g.*, 5, 27, 33). Therefore, we used t-Bid as a unique experimental tool to distinguish the locations of GRx1 and GRx2 in the mitochondria.

t-BID TREATMENT OF MITOCHONDRIA REVEALS UNIQUE LOCATIONS OF GRx1 AND GRx2

Analysis of isolated mitochondrial fractions that were *not* treated with t-Bid revealed GRx1 content harvested from the mitochondria into the *mitochondrial extract*, but no GRx1 was detected in the *supernatant* of the untreated mitochondria (Fig. 1A, *minus t-Bid*). This result documents that the GRx1 content associated with the mitochondria cannot be ascribed to cytosolic contamination. Lack of significant cytosolic contamination in the mitochondrial preparations was also documented separately by measurements of lactate dehydrogenase activity (cytosolic marker) in the supernatant fractions of untreated mitochondria (*i.e.*, ≤0.2% of total LDH activity was observed in the standard preparations of mitochondria). Additional purification by Percoll gradient centrifugation further diminished LDH contamination, but the specific mitochondrial content of GRx1 was unchanged (data not shown). In remarkable contrast, after treatment of the mitochondria with t-Bid, apparently all of the GRx1 content was now released to the *supernatant* (see Fig. 1A, *plus t-Bid*), and none was detected in the *mitochondrial extract* or the *residue*. These results indicate that GRx1 is localized exclusively in the intermembrane space of the mitochondria. Quantitative mass balance analysis of multiple preparations of rat liver mitochondria reinforces this conclusion (Table 1). Essentially all of the GRx1 found in the mitochondria is releasable by t-Bid treatment. It was confirmed in separate experiments that the amount of deglutathionylase activity released from 5 mg of mitochondria by t-Bid corresponded quantitatively to the amount of GRx1 protein released (activity predicted, 1.4 mU; and activity observed, 1.3 mU). Thus, GRx1 is released from the intermembrane space as the fully active enzyme. Furthermore, we observed the same qualitative and quantitative pattern of results for subsarcolemmal mitochondria isolated from Fischer 344 rat hearts (Fig. 2A and Table 1). These comparable results for mitochondria isolated from different tissues and different strains of rats support the conclusion that localization of GRx1 to the mitochondrial intermembrane space is a generalizable phenomenon.

Opposite results of t-Bid treatment were observed for GRx2. Instead of releasing the protein to the supernatant, t-Bid had no effect on the association of GRx2 with the mitochondrial fraction (see Figs. 1 and 2B, and Table 1). These results support the conclusion that GRx2 is localized in the mitochondrial ma-

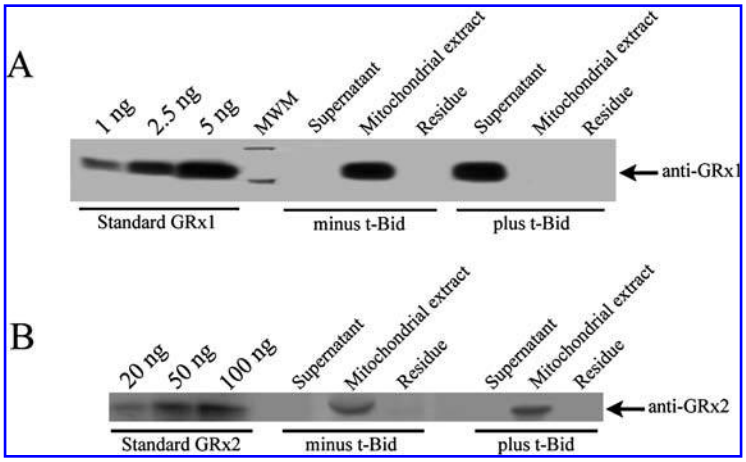

FIG. 1. Localization of GRx1 and GRx2 in mitochondrial preparations from Sprague–Dawley rat livers. Liver mitochondria (2 mg) were incubated for 60 min at 37°C in the absence or presence of t-Bid (45 n*M*) in separate centrifuge tubes (total volume, 100 μl). Sequential mitochondrial fractions were prepared as described under *Appendix notes* and were used to obtain the samples designated "Supernatant," "Mitochondrial Extract," and "Residue," respectively, before SDS-PAGE and Western blot analysis with the appropriate primary antibodies as follows. (**A**) Liver mitochondrial protein fractions probed with anti-GRx1 antibody. (**B**) Liver mitochondrial protein fractions probed with anti-GRx2 antibody.

trix, consistent with expression of the protein with a leader sequence that directs it to the matrix (11, 25).

CONTROL EXPERIMENTS CONFIRM THE SELECTIVE EFFECT OF t-BID ON THE OUTER MITOCHONDRIAL MEMBRANE

Several positive and negative control experiments were conducted to verify our conclusions about the differential localization of GRx1 and GRx2. First, we documented the effect of t-Bid treatment on the fractionation of adenylate kinase 2 (AK-2), a standard indicator for the intermembrane space (27). Figure 3 displays the expected pattern for AK-2 fractionation: it is fully released from the mitochondria by t-Bid, recapitulating the data for GRx1 and reinforcing the conclusion that GRx1 is localized as a soluble protein in the intermembrane space. Second, as reported previously by others (30), we observed t-Bid–dependent release of cytochrome *c* from the intermembrane space (see Fig. 3A2 and B2). Unlike GRx1 and AK-2, not all of the cytochrome *c* was released by the t-Bid treatment, consistent with results from previous studies that distinguished soluble and sequestered forms of cytochrome *c* in the IMS (33). Use of higher ionic strength buffer or higher concentration of t-Bid (or both) can lead to complete release of cytochrome *c* (34). This distinction makes GRx1 more akin to AK-2, smac/DIABLO, and other IMS proteins that are fully releasable by t-Bid (33).

To confirm that the t-Bid treatment and selective release of proteins from the intermembrane space did not disrupt the outer mitochondrial membrane integrity or affect mitochondrial matrix proteins, we measured specific mitochondrial marker enzyme activities. Assay of cytochrome *c* oxidase to assess outer membrane integrity showed no difference in the percentage of leakiness of the outer mitochondrial membrane between untreated and t-Bid–treated liver mitochondria [20.3 ± 3.8% and 20.0 ± 3.1%, respectively (mean ± SEM for three separate experiments)]. Assays of the mitochondrial matrix marker citrate synthase were performed to test, independent of GRx2 analysis, whether t-Bid treatment affected the intactness of the matrix compartment. These analyses showed no difference in total specific activity between untreated mitochondria and mitochondria treated with t-Bid [244.9 ± 15.4 and 263.5 ± 26.7 nmol/min/mg protein, respectively (mean ± SEM for four separate experiments)], in-

TABLE 1. DENSITOMETRIC ANALYSIS OF GRx1 AND GRx2 CONTENTS IN MITOCHONDRIA AND SUBMITOCHONDRIAL FRACTIONS

	Sprague-Dawley rat liver mitochondria			*Fischer 344 Rat Heart subsarcolemmal mitochondria*		
	Untreated mitochondria	*t-Bid–treated mitochondria*	*t-Bid releasable*	*Untreated mitochondria*	*t-Bid–treated mitochondria*	*t-Bid releasable*
GRx1	2.40 ± 0.16	N.D.	2.26 ± 0.16	0.91 ± 0.12	N.D.	0.89 ± 0.09
GRx2	17.0 ± 0.7	17.8 ± 0.5	N.D.	23.8 ± 0.8	24.2 ± 0.5	N.D.

Total amounts of specific GRx1 or GRx2 protein present in each of the samples analyzed by Western blot analysis were calculated according to their densitometric band intensities relative to the band intensities for known amounts of purified human GRx1 or human GRx2 standards run simultaneously on the same SDS–PAGE gels. The reported values (expressed as ng GRx1 or GRx2/mg of mitochondrial protein) represent the mean ± SEM for three separate experiments. N.D., not detectable. Note: Because the rat GRx1 and GRx2 proteins were analyzed according to their immunoreactivity with anti-human GRx1 and anti-human GRx2 antibodies, and compared with the immunoreactivity of human protein standards (GRx1 and GRx2), the values in the table must be considered relative rather than absolute values. Nevertheless, the cross-immunoreactivity is expected to be high because of high homology between the rodent and human enzymes and the use of polyclonal antibodies.

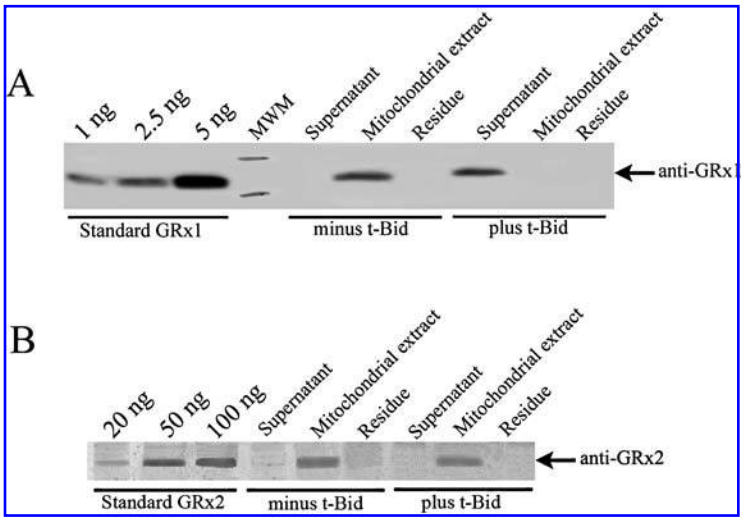

FIG. 2. Localization of GRx1 and GRx2 in subsarcolemmal mitochondrial preparations from Fischer 344 rat hearts. Heart SSM (2 mg) were incubated for 60 min at 37°C in the absence or presence of t-Bid (45 n*M*) in separate centrifuge tubes (total volume, 100 μl). Sequential mitochondrial fractions were prepared as described under *Appendix notes* and were used to obtain the samples designated "Supernatant," "Mitochondrial Extract," and "Residue," respectively, before SDS-PAGE and Western blot analysis with the appropriate primary antibodies as follows. (**A**) Heart SSM protein fractions probed with anti-GRx1 antibody. (**B**) Heart SSM protein fractions probed with anti-GRx2 antibody.

dicating that t-Bid did not disrupt the inner mitochondrial membrane and allow release of the citrate synthase enzyme. Our results confirm previous observations that t-Bid mediates limited permeabilization of the outer mitochondrial membrane, as documented by lack of accessibility of inner membrane–associated respiratory complexes to externally added cytochrome *c* (19), and ultrastructural studies that did not distinguish t-Bid–treated from untreated mitochondria (34).

SEPARATE LOCALIZATION OF GRx1 AND GRx2 SUGGESTS COMPARTMENTAL REDOX REGULATION IN MITOCHONDRIA

The mitochondrion is an exceptional example of a subcellular organelle whose function is closely linked to maintainance

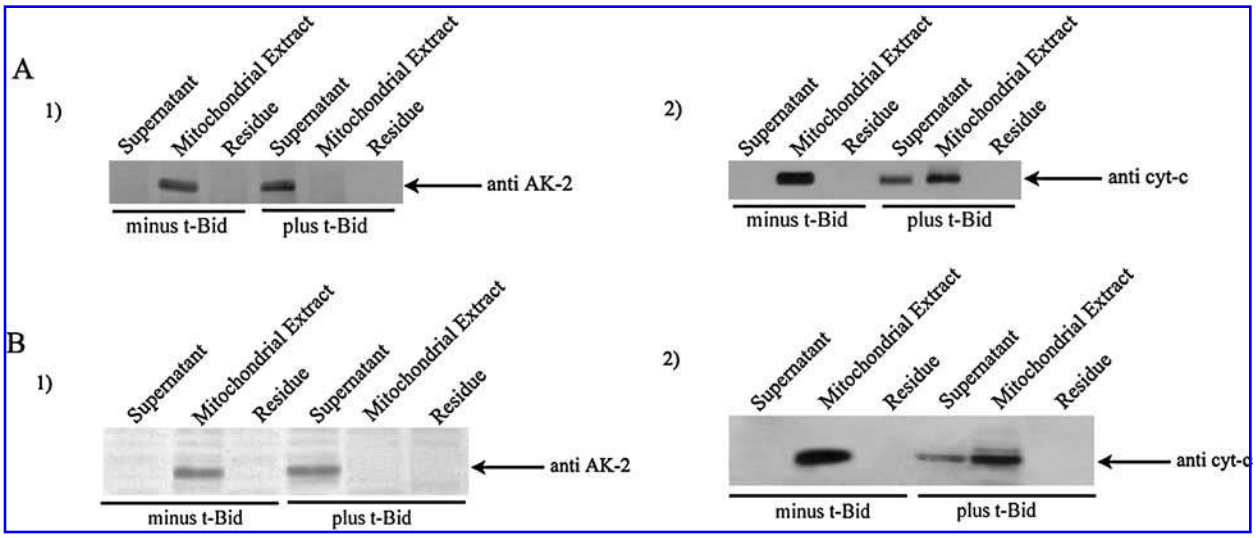

FIG. 3. t-Bid release of known mitochondrial intermembrane space proteins. (**A**) Liver mitochondria (2 mg) were incubated for 60 min at 37°C in the absence or presence of t-Bid (45 n*M*) in separate centrifuge tubes (total volume, 100 μl). Sequential mitochondrial fractions were prepared as described under *Appendix notes* and were used to obtain the samples designated "Supernatant," "Mitochondrial Extract," and "Residue," respectively, before SDS-PAGE and Western blot analysis with the appropriate primary antibodies as follows. All of the respective liver mitochondrial fractions were diluted 1:40 with 1x SDS-PAGE sample buffer before SDS-PAGE and Western blot analysis with either (1) anti-AK-2 antibody or (2) anti-cyt c antibody. (**B**) Heart SSM (2 mg) were incubated for 60 min at 37°C in the absence or presence of t-Bid (45 n*M*) in separate centrifuge tubes (total volume, 100 μl). Sequential mitochondrial fractions were prepared as described under *Appendix notes* and were used to obtain the samples designated "Supernatant," "Mitochondrial Extract," and "Residue," respectively, before SDS-PAGE and Western blot analysis with the appropriate primary antibodies as follows. All of the respective heart SSM mitochondrial fractions were diluted 1:40 with 1x SDS-PAGE sample buffer before SDS-PAGE and Western blot analysis with either (1) anti-AK-2 antibody or (2) anti-cyt c antibody.

of redox balance. Although most cellular glutathione (GSH) is in the cytoplasm, a distinctly regulated pool is present in mitochondria (21), and a large number of mitochondrial proteins, including dehydrogenases and transport ATPases, contain critical sulfhydryl groups that must be maintained in the reduced form for proper function. Moreover, changes in mitochondrial GSH status have been associated with activation of signaling pathways and expression of genes that regulate apoptosis (19) and cell growth and differentiation (10). GSH participates in sulfhydryl homeostasis *via* scavenging reactive species (oxidants, radicals, electrophiles) or through thiol-disulfide exchange reactions that are catalyzed by the glutaredoxin enzymes. As mitochondria are the primary intracellular sites of oxygen consumption, they also represent primary sites of generation of reactive oxygen species (ROS). Different regions of the electron-transport chain may produce ROS in a vectorial fashion, so that inhibition of complex I increases ROS production in the direction of the matrix (4), whereas inhibition of complex III increases ROS directed toward the intermembrane space (1, 4). Consequently, vital sulfhydryl-sensitive proteins in the subcompartments of the mitochondria may be subject to different oxidative stresses that lead to protein-SSG formation, requiring local homeostatic regulation by GRx1 (intermembrane space) and GRx2 (matrix).

CONCLUSIONS AND OPEN QUESTIONS

GRx1 is found preponderantly in the cytosol of cells; however, several previous studies implicated its presence in mitochondria, and we have now documented its exclusive presence in the intermembrane space, distinct from GRx2, which is localized solely to the matrix of mitochondria. The ability to segregate GRx1 from GRx2 while maintaining the structural integrity of the mitochondria provides the opportunity to distinguish the relative contributions of the two isoforms to sulfhydryl homeostasis of specific components of the mitochondria. Thus, GRx1 in the intermembrane space and GRx2 in the matrix are likely to regulate different functions of the mitochondria *via* reversible *S*-glutathionylation. For example, the distinct localization of GRx1 and its release *via* a mechanism that mimics the initiation of apoptosis suggests a potential role for GRx1 in redox regulation of apoptosis, as well as regulation of distinct transport processes peculiar to the intermembrane space. GRx2 is poised for redox regulation of matrix proteins, as well as participation in maintenance of iron-sulfur cluster proteins.

Many intriguing questions are related to the compartmentation of GRx1 and GRx2 in mitochondria and form the basis for further study [*e.g.*, (a) whether a dynamic equilibrium exists for GRx1 between cytosol and the intermembrane space, and how this distribution is affected by redox signaling (in the cytosol or the mitochondria), or by aging and/or diseases that propagate oxidative stress conditions; (b) whether IMS-GRx1 has a mitochondrial localization sequence that directs it to the intermembrane space; (c) whether GRx1 participates in the release and/or activation of other components of the apoptosome; and (d) whether the deglutathionylase activity of GRx2 is regulated in the mitochondrial matrix by a redox-sensitive dynamic equilibrium between the monomeric (active) and dimeric-iron-sulfur (inactive) forms of the enzyme.

APPENDIX

Notes

1. Animals and mitochondrial isolation: Male Sprague–Dawley rats (200–400 g) or Fisher 344 rats (6 months, 350–450 g) were obtained from the Animal Care and Use Facility of Case Western Reserve University. Hearts from the Fischer rats were excised and placed into buffer A [100 m*M* KCl, 50 m*M* 3-(*N*-morpholino) propanesulfonic acid (MOPS), 1 m*M* EGTA, 5 m*M* $MgSO_4$, and 1 m*M* ATP; pH 7.4] at 4°C. Cardiac mitochondria were isolated using the procedure of Palmer *et al*. (31). In brief, heart tissue was finely minced and placed in buffer A, containing 0.2% bovine serum albumin, and homogenized with a polytron tissue processor (Brinkman Instruments, Westbury, NY) for 2.5 s at a rheostat setting of 6. The polytron homogenate was centrifuged at 500 *g*, the supernatant saved for isolation of subsarcolemmal mitochondria (SSM), and the pellet washed. The combined supernatants were centrifuged at 3,000 *g* to sediment SSM. SSM were washed twice and then suspended in KME buffer (80 m*M* KCl, 50 m*M* MOPS, and 0.5 m*M* EGTA). Mitochondrial protein concentration was measured by the Lowry method, using bovine serum albumin as a standard. Livers were excised from the Sprague–Dawley rats, and liver mitochondria were prepared according to the procedure described by Hoppel *et al.* (15). To document lack of significant cytosolic contamination of the isolated mitochondria, LDH activity was measured according to pyruvate-dependent NADH oxidation by standard spectrophotometric assay (ΔA_{340nm}) (24).

2. Assays of mitochondrial oxidative phosphorylation: Oxygen consumption by mitochondria was measured using a Clark-type oxygen electrode at 30°C (31). Mitochondria were incubated in 80 m*M* KCl, 50 m*M* MOPS, 1 m*M* EGTA, 5 m*M* KH_2PO_4, and 1 mg defatted dialyzed bovine serum albumin/ml at pH 7.4. Glutamate (complex I substrate, 20 m*M*) was used as substrate, and the following measurements were made: state 3 (0.2 m*M* ADP-stimulated), state 4 (ADP-limited) respiration, respiratory control ratio, and maximal ADP-stimulated respiration (2 m*M* ADP). The respiratory control ratio and ADP/O ratio were determined to be typical of intact functional mitochondria before using the mitochondria for further assays (31). Mitochondria always were used within 6 to 8 h after isolation from tissue. Endogenous substrates were depleted by addition of 0.1 m*M* ADP before addition of glutamate.

3. Assays of cytochrome oxidase and citrate synthase activities: These enzyme activities were measured for detergent-solubilized, freshly isolated rat liver mitochondria or heart SSM at 37°C using previously described methods (15, 31). Outer mitochondrial membrane integrity was assessed in the presence and absence of detergent, sodium dodecyl maltoside (0.05%, vol/vol). Cytochrome *c* oxidase is present in the inner mitochondrial membrane, and it normally accepts electrons from cytochrome *c* contained within the intermembrane space. The ability of reduced cytochrome *c*, added outside the mitochondria, to support cytochrome *c* oxidase activity is therefore a measure of integrity of the outer mitochondrial membrane (OMM). If the membrane were intact, then externally added cytochrome *c* would have no effect. Thus, the difference in cytochrome oxidase activity with and without detergent reflects the integrity of the outer membrane (15). Similarly citrate synthase activity was used as the matrix marker to serve as an indicator for inner mitochondrial membrane integrity.

4. Treatment with t-Bid and preparation of mitochondrial extracts for SDS-PAGE: Freshly isolated liver mitochondria or heart SSM were prepared from Sprague–Dawley or Fischer 344 rats, respectively. The respective mitochondrial preparations were diluted with MSM buffer (220 m*M* mannitol, 70 m*M* sucrose, 5 m*M* MOPS, pH 7.4) or buffer A to a concentration of 20 mg/ml (total volume, 100 μl). These mitochondrial samples were incubated at 37°C with or without t-Bid (45 n*M*)

for an hour (20) and centrifuged at 3,500 *g* for 10 min at 4°C. The resulting supernatant is referred to as "supernatant," representing contents released from the intermembrane space. The pellets were resuspended in 100 μl of MSM buffer, sonicated thoroughly on ice, and centrifuged at 16,000 *g* for 15 min at room temperature. The resultant supernatants were heat treated at 60°C for 2 min and then placed on ice. After chilling, the samples were centrifuged at 16,000 *g* for 20 min. The resultant supernatant is referred to as "mitochondrial extract," representing the total amount of specific proteins (GRx1, GRx2, AK-2, cyt c) harvested from mitochondria before or after t-Bid treatment. The residual pellets obtained after centrifugation of the mitochondrial extracts are referred to as "residue," representing any material not harvested from the mitochondria. These descriptive terms are used as headings in Figs. 1 and 2.

5. Western blot analyses: Aliquots of the samples obtained from the variously processed mitochondria (described above) were combined with concentrated stock SDS-PAGE sample buffer to achieve the final composition [1× sample buffer = 0.125 *M* Tris-HCl, pH 6.8, 5% glycerol, 2.5% SDS (wt/vol), and 0.25% bromophenol blue containing 5 m*M* DTT]. The resultant mixtures were then heated at 95°C for 10 min. Iodoacetamide solution was added to achieve a final concentration of 20 m*M* to derivatize cysteine-SH groups. These samples were loaded onto 12% acrylamide large-format gels to accommodate sample volumes up to 250 μl. The proteins were transferred onto PVDF membranes using a semidry transferring apparatus at 6 V for 30 min followed by 24 V for 1 h and 30 min. The resulting blots were blocked overnight in Tris-buffered saline, pH 7.4, containing 5% nonfat milk. The blots were first incubated with the appropriate primary antibody preparation: rabbit anti-human GRx1 polyclonal antibody (13) (1:1,000 dilution) for 2 h, or rabbit anti-human polyclonal GRx2 antibody (11) (1:1,000 dilution), or rabbit anti-adenylate kinase 2 polyclonal antibody (Abcam; 1:1,000 dilution), or mouse anti-cytochrome *c* monoclonal antibody (BD Pharmingen, San Diego, CA; 1:1,000 dilution). The blots were then incubated with peroxidase conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA; 1:10,000 dilution) for 1 h before ECL detection to visualize the bands, according to the manufacturer's protocol (PerkinElmer Life Sciences, Woodbridge, ON, Canada). Blots probed with anti-GRx2 antibody were incubated with anti-rabbit fluorescein secondary antibody (1:1,000 dilution) followed by anti-fluorescein tertiary antibody containing alkaline phosphatase (1:1,000 dilution) before ECF development, according to manufacturer's protocol (Amersham Biosciences, Piscataway, NJ).

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ABBREVIATIONS

AK-2, adenylate kinase 2; cyt c, cytochrome *c*; GRx, glutaredoxin; GSH, glutathione, IMS, intermembrane space; OMM, outer mitochondrial membrane; TRx, thioredoxin; t-Bid, truncated Bid.

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Address reprint requests to:
Dr. John J. Mieyal
2109 Adelbert Road
Department of Pharmacology, School of Medicine
Case Western Reserve University
Cleveland, OH 44106-4965

E-mail: jjm5@cwru.edu

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