Reversible Inactivation of α-Ketoglutarate Dehydrogenase in Response to Alterations in the Mitochondrial Glutathione Status†

Amy C. Nulton-Persson,‡ David W. Starke,§ John J. Mieyal,§ and Luke I. Szweda*,‡

Department of Physiology and Biophysics and Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

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ABSTRACT: In a previous study, we found that treatment of rat heart mitochondria with H_2O_2 resulted in a decline and subsequent recovery in the rate of state 3 NADH-linked respiration. These effects were shown to be mediated by reversible alterations in NAD(P)H utilization and in the activities of specific Krebs cycle enzymes α-ketoglutarate dehydrogenase (KGDH) and succinate dehydrogenase. The purpose of the current study was to examine potential mechanism(s) by which H_2O_2 reversibly alters KGDH activity. We report here that inactivation is not simply due to direct interaction of H_2O_2 with KGDH. In addition, incubation of mitochondria with deferroxamine, an iron chelator, or 1,3-dimethyl-2-thiourea, an oxygen radical scavenger, prior to addition of H_2O_2 did not alter the rate or extent of inactivation. Thus, inactivation does not appear to involve a more potent oxygen radical formed upon metal-catalyzed oxidation. Inactive KGDH from H_2O_2-treated mitochondria was reactivated with dithiothreitol, implicating oxidation of a protein sulfhydryl(s). However, the thioredoxin system had no effect, indicating that enzyme inactivation is not due to the formation of intra- or intermolecular disulfide(s) or a sulfenic acid. Upon incubation of mitochondria with H_2O_2, reduced GSH levels fell rapidly prior to enzyme inactivation but recovered at the same time as enzyme activity. Importantly, treatment of inactive KGDH with glutaredoxin facilitated the GSH-dependent recovery of KGDH activity. Glutaredoxin is characterized as a specific and efficient catalyst of protein deglutathionylation. Thus, the results of the current study indicate that KGDH activity appears to be modulated through enzymatic glutathionylation and deglutathionylation. These studies demonstrate a novel mechanism by which KGDH activity and mitochondrial function can be modulated by redox status.

Emerging evidence indicates that certain pro-oxidants can act to modulate cellular function. Production of reactive oxygen species has been shown to increase in response to activation of various receptors. This increase has been linked to the subsequent activation of certain signal transduction pathways. Purported mechanisms by which oxidant species modulate protein function include reversible oxidation of key protein sulfhydryl residues and alterations in the interactions between redox sensitive molecules and target proteins (*1*−*8*). The mitochondrial electron transport chain has long been recognized as a site of $O_2^{\bullet-}$ production under both normal and pathophysiologic conditions (*9*, *10*). To date, the assumption has been that this is an unfortunate consequence of electron transport that necessitates scavenging mechanisms for clearing potentially toxic pro-oxidants. However, a futile cycle such as this carries a high metabolic price. These considerations together with the growing body of evidence implicating oxygen radicals as regulators of cellular function suggest a physiological role for free radicals produced within mitochondria.

In a previous study, we found that the rate of mitochondrial respiration declines and subsequently recovers when intact, respiring mitochondria are treated with micromolar concentrations of H_2O_2 (*11*). As shown in our earlier work, this response to H_2O_2 was mediated, in part, by reversible alterations in the activities of α-ketoglutarate dehydrogenase (KGDH)[1] and succinate dehydrogenase (SDH) (*11*). Inactivation and reactivation of KGDH and SDH required mitochondria to remain intact with respiratory substrates present. In addition, removal of H_2O_2 alone was not sufficient for reactivation (*11*). These observations suggest that KGDH and SDH are responsive to the redox status of the mitochondria. While it is well-established that proteins can be oxidatively inactivated (*12*), the reversible nature of the inactivation of key Krebs cycle enzymes by pro-oxidants which are produced by mitochondria suggests potential regulatory roles for these species (*11*). It is therefore critical to understand the mechanism by which enzyme activity is reversibly altered in response to H_2O_2 to gain insight into the metabolic relevance of these processes.

Alterations in KGDH activity are of particular interest given that numerous degenerative processes associated with oxidative stress are accompanied by a loss in KGDH activity (*13*−*17*). Thus, the purpose of this study was to examine

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* To whom correspondence should be addressed: Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4970. Telephone: (216) 368-0035. Fax: (216) 368-1693. E-mail: Lxs54@po.cwru.edu.

‡ Department of Physiology and Biophysics.

§ Department of Pharmacology.

[1] Abbreviations: KGDH, α-ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; GRx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; SD, standard deviation.

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the mechanism by which H_2O_2 reversibly alters KGDH activity. To accomplish this, solubilized mitochondria and pure KGDH were treated with specific oxidants and reducing systems to provide evidence of the nature of inactivation and reactivation. In addition, the levels of various pro- and antioxidant species were monitored in H_2O_2-treated mitochondria to define likely components involved in alterations in enzyme activity. The results demonstrate that H_2O_2 mediates the modification of sulfhydryl group(s) on KGDH. Reactivation by glutaredoxin but not thioredoxin indicates that KGDH is reversibly glutathionylated in response to H_2O_2. The mechanism as well as the specific and discreet nature of the modification is discussed with regard to the physiological significance of H_2O_2-induced alterations in KGDH activity.

MATERIALS AND METHODS

Reagents and Proteins. Thioredoxin, thioredoxin reductase, and glutathione (reduced and oxidized) were obtained from Sigma. Recombinant human glutaredoxin was purified from *Escherichia coli* as described previously (*18*).

Isolation of Subsarcolemmal Mitochondria from Rat Heart. Sprague-Dawley rats (200−300 g) obtained from Zivic Miller Laboratories were anesthetized with sodium pentobarbital and decapitated. Hearts were removed and immediately immersed and rinsed in ice cold homogenization buffer containing 180 mM KCl, 5.0 mM MOPS, and 2.0 mM EDTA at pH 7.4. Hearts (0.9−1.1 g) were then minced and homogenized in 20 mL of homogenization buffer with a Polytron homogenizer (low setting, 2 s). The homogenate was centrifuged at 500*g* for 5 min (5 °C), and the supernatant was filtered through cheesecloth. The mitochondrial pellet was obtained upon centrifugation of the supernatant at 5000*g* for 10 min (5 °C). After two rinses with ice cold buffer, the mitochondria were resuspended in homogenization buffer to a final concentration of approximately 35.0 mg/mL. Protein determinations were made using the bicinchroninic acid (BCA) method (Pierce), with bovine serum albumin as a standard.

Incubation of Intact Mitochondria with H_2O_2 and tert-Butylhydroperoxide. Mitochondria were diluted to a concentration of 0.25 mg/mL in buffer composed of 125 mM KCl and 5.0 mM KH_2PO_4 at pH 7.25. Respiration was initiated by the addition of 15 mM α-ketoglutarate and allowed to proceed for 1.0 min. State 3 respiration was then induced by addition of 2.0 mM ADP. One minute after initiation of state 3 respiration, H_2O_2 or *tert*-butylhydroperoxide (50 μM) was added. All incubations were performed at room temperature.

Assay of α-Ketoglutarate Dehydrogenase Activity. KGDH activity was evaluated after mitochondria had been subjected to various conditions. Mitochondria were diluted to a concentration of 0.05 mg/mL in 25.0 mM KH_2PO_4 and 0.5 mM EDTA at pH 7.25 and solubilized with 0.1% Triton X-100 (Sigma). KGDH activity was assayed spectrophotometrically as the rate of NADH production (340 nm, $\epsilon_{NADH} = 6200$ M^{-1} cm^{-1}) upon addition of 5.0 mM $MgCl_2$, 40.0 μM rotenone, 2.5 mM α-ketoglutarate, 0.1 mM CoA, 0.2 mM thiamine pyrophosphate, and 1.0 mM NAD^+ to solubilized mitochondria (0.05 mg/mL mitochondrial protein) (*11*).

Assay of H_2O_2. Following various treatments, intact mitochondria were diluted to a concentration of 0.05 mg/mL in 125 mM KCl and 5.0 mM KH_2PO_4 at pH 7.25 containing 500 μM *p*-hydroxyphenylacetic acid. The increase in hydroxyphenylacetate fluorescence was monitored spectrofluorometrically (Shimadzu RF-5301PC), at excitation and emission wavelengths of 320 and 425 nm, respectively, with slit widths of 5.0 nm, upon addition of 13 units/mL horseradish peroxidase (*19*). The corresponding fluorescence intensities were converted to H_2O_2 concentration by comparison to a standard curve.

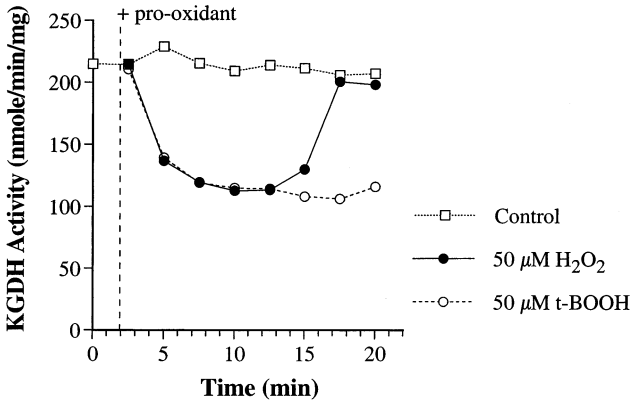

FIGURE 1: Time-dependent alterations in KGDH activity upon treatment of mitochondria with H_2O_2 or *tert*-butylhydroperoxide. Intact rat heart mitochondria (0.25 mg/mL) were incubated with 15 mM α-ketoglutarate (0 min) as a respiratory substrate and 2.0 mM ADP (1.0 min) to initiate state 3 respiration. At 2.0 min, mitochondria either were left untreated or were treated with 50 μM H_2O_2 or *tert*-butylhydroperoxide. At the times indicated on the abscissa, mitochondria were solubilized with 0.1% Triton X-100 and KGDH activity was measured spectrophotometrically.

Determination of Intramitochondrial Reduced Glutathione Levels. Mitochondria were solubilized with 0.1% Triton X-100 and diluted with an equal volume of a mobile phase containing 10 mM trichloroacetic acid and 69 mM monochloroacetic acid (pH 2.72, adjusted with NaOH). The sample was then sonicated for 30 s in a sonicating water bath and centrifuged at 15000*g* for 5 min. The supernatant was analyzed by HPLC (Hewlett-Packard series 1050). GSH was resolved on a C_{18} reverse phase column (Vydac) and detected using an electrochemical detector containing a glassy carbon working electrode (−0.75 V) and an AgCl reference electrode. A standard curve was generated using pure authentic GSH.

RESULTS

Effect of H_2O_2 and tert-Butylhydroperoxide on KGDH Activity. Intact respiring mitochondria were treated with 50 μM H_2O_2 or *tert*-butylhydroperoxide. At the indicated times (Figure 1), mitochondria were solubilized and KGDH activity was measured to determine whether the two pro-oxidants exerted differential effects. Over the course of 20 min, KGDH activity in mitochondria treated with H_2O_2 declined and recovered as previously shown (*11*). However, while *tert*-butylhydroperoxide induced a similar decline in activity, no recovery was observed (Figure 1). Extension of the incubation time to 60 min failed to show reversal of the inhibitory effect of *tert*-butylhydroperoxide on KGDH activity (results not shown). Addition of deferroxamine (from 0 to 5 mM, 5.0 min), an iron chelator, or 1,3-dimethyl-2-thiourea (from 0 to 5 mM, 5.0 min), an oxygen radical scavenger, prior to treatment of mitochondria with H_2O_2 or *tert*-butylhydroper-

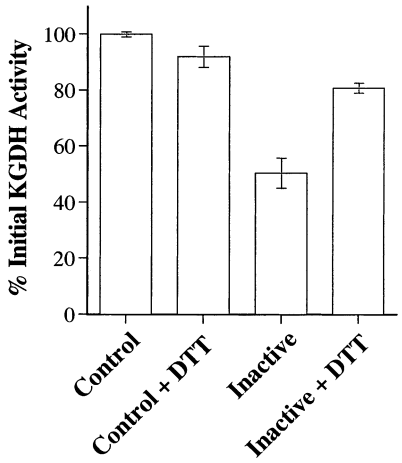

FIGURE 2: Effect of DTT on KGDH inactivated by treatment of mitochondria with H_2O_2. Intact rat heart mitochondria (0.25 mg/mL) were incubated with 15 mM α-ketoglutarate (0 min) as a respiratory substrate and 2.0 mM ADP (1.0 min) to initiate state 3 respiration. At 2.0 min, mitochondria either were left untreated (Control) or were treated with 50 μM H_2O_2 (Inactive). At 7.5 min, mitochondria were solubilized with 0.1% Triton X-100 and either left untreated or treated with 100 mM dithiothreitol (DTT) for 2.0 min as indicated. Samples were then diluted to 0.05 mg/mL and assayed for KGDH activity spectrophotometrically. Values represent means ($n = 3$) ± SD.

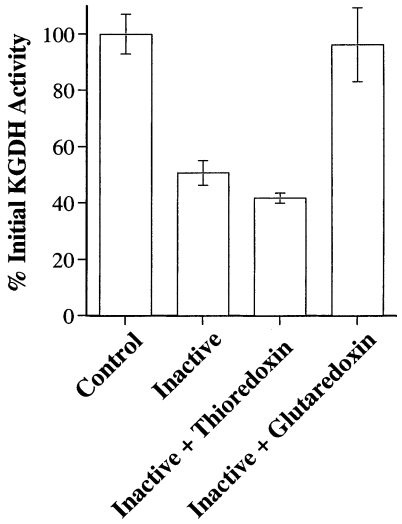

FIGURE 3: Effect of the thioredoxin and glutaredoxin reducing systems on KGDH inactivated by treatment of mitochondria with H_2O_2. Intact rat heart mitochondria (0.25 mg/mL) were incubated with 15 mM α-ketoglutarate (0 min) as a respiratory substrate and 2.0 mM ADP (1.0 min) to initiate state 3 respiration. At 2.0 min, mitochondria either were left untreated (Control) or were treated with 50 μM H_2O_2 (Inactive). At 7.5 min, mitochondria were solubilized with 0.1% Triton X-100 and treated with the thioredoxin (5.0 μM thioredoxin, 5.0 μM thioredoxin reductase, and 50 μM NADPH) or the glutaredoxin reducing system (0.05 unit/mL = 0.04 μM glutaredoxin and 0.5 mM GSH) for 2.5 min. Mitochondria were subsequently diluted to 0.05 mg/mL, and KGDH activity was assayed spectrophotometrically. Values represent means ($n = 3$) ± SD.

oxide did not alter the rate or extent of inactivation or reactivation. In addition, neither commercially purified KGDH nor KGDH from solubilized mitochondria was inactivated by H_2O_2 or *tert*-butylhydroperoxide (5.0 mM, 5.0 min). Thus, inactivation does not appear to be due to direct interaction of the enzyme with the peroxide or to a more potent oxygen radical formed upon metal-catalyzed oxidation.

Effect of DTT on H_2O_2 and tert-Butylhydroperoxide-Inactivated KGDH. Likely sites of oxidative modification on a protein are sulfhydryl-containing amino acids. DTT will reduce sulfhydryl residues that are oxidized to either a disulfide (intra- or intermolecular or mixed) or a sulfenic acid. Higher oxidation states cannot be reversed by DTT. To evaluate whether H_2O_2 or *tert*-butylhydroperoxide induces sulfhydryl oxidation on KGDH, H_2O_2- and *tert*-butylhydroperoxide-treated mitochondria were solubilized and subsequently treated with DTT. DTT was able to largely reverse both H_2O_2-mediated (Figure 2) and *tert*-butylhydroperoxide-mediated (not shown) inactivation. This indicates that modification of a sulfhydryl residue(s) on KGDH is, in large part, responsible for inactivation. Interestingly, DTT-induced recovery was not observed at concentrations of <10 mM and was not improved by extending the incubation time (>5.0 min).

Effect of the Thioredoxin Reducing System on KGDH. To further explore the nature of KGDH inactivation and reactivation, the ability of a disulfide/sulfenic acid reducing system to reactivate KGDH was assessed. Thioredoxin in combination with NADPH and thioredoxin reductase will reduce oxidized sulfhydryl residues within a protein in either a sulfenic acid configuration or an intra- or intermolecular disulfide but is inefficient in removing glutathione from protein sulfhydryls. Intact, respiring mitochondria were incubated in the presence or absence of H_2O_2 (Figure 3) or *tert*-butylhydroperoxide until maximal inactivation was achieved. Mitochondria were then solubilized and treated with thioredoxin, thioredoxin reductase, and NADPH. The thioredoxin reducing system exhibited no effect on inactive KGDH, indicating that inactivation of KGDH is not due to formation of a disulfide or a sulfenic acid. This is in keeping with the inability of H_2O_2 or *tert*-butylhydroperoxide to directly inactivate KGDH given the fact that these pro-oxidants have the potential to catalyze the formation of disulfide bonds.

Assessing KGDH as a Protein Mixed Disulfide with Glutathione. An alternative mechanism by which KGDH may be inactivated in a sulfhydryl-dependent fashion is through glutathionylation of the protein. We therefore undertook a series of experiments with the goal of obtaining immunochemical and/or mass spectroscopic evidence for glutathionylation of KGDH. KGDH is comprised of multiple copies of three subunits, α-ketoacid decarboxylase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoamide dehydrogenase (E3). Both E2 and E3 contain sulfhydryl residues which cycle between reduced and oxidized states during enzyme catalysis and therefore represent potential sites of glutathionylation (*20*, *21*). Following treatment of intact respiring cardiac mitochondria with H_2O_2, we performed nonreducing one-dimensional (1D) (10−50 μg of solubilized mitochondrial protein/lane) and two-dimensional (2D) (100−500 μg of delipidated mitochondrial protein/experiment) gel electrophoresis and Western blot analyses utilizing antibodies raised to glutathione. The appearance of glutathionylated proteins, as assessed by these methods, was not observed upon H_2O_2 treatment. The lack of detectable primary antibody binding could be due to insufficient sensitivity and/or to certain intrinsic properties of KGDH which make it difficult to use existing methodologies required to assess the glutathionylation status of a protein. (1) KGDH readily

aggregates under nonreducing conditions necessary for analysis by 1D and 2D gel electrophoresis. This was particularly true for mitochondria treated with H_2O_2 and is likely due to the presence of numerous sulfhydryl residues normally involved in enzyme catalysis and the hydrophobic nature of certain interactions between subunits. KGDH is also associated with the inner mitochondrial membrane, further complicating extraction and analysis. (2) The maximum level of inactivation achieved is typically 40%. In addition, KGDH is composed of multiple copies of three interacting subunits. Therefore, the level of modification relative to the degree of inactivation may not be stoichiometric, even for a given subunit, making it difficult to detect modified protein with the low sensitivity of commercially available antibodies to glutathionylated protein. Use of greater quantities of protein led to poor resolution and enhanced aggregation. These technical difficulties could not be overcome by immunopurification of the enzyme, as quantitative extraction of KGDH from H_2O_2-treated cardiac mitochondria was not accomplished utilizing existing antibodies to lipoic acid or to the E3 subunit of KGDH under nonreducing conditions required for further analysis. Finally, experiments aimed at introducing biotinylated GSH into intact cardiac mitochondria prior to H_2O_2 treatment, thereby providing a means for enhanced immunochemical detection of protein−glutathione adducts, were performed. However, consistent with previous findings that biotinylated GSH is impermeable to the plasma membrane (*22*), sufficient quantities of the compound could not be introduced into mitochondria. Clearly, a variety of technical difficulties must be overcome to obtain direct evidence for glutathionylation of KGDH.

Glutaredoxin is a specific and efficient catalyst for deglutathionylating proteins (*18*, *23*). We therefore used this enzyme to assess whether KGDH was glutathionylated following H_2O_2 treatment. Intact, respiring mitochondria were incubated in the presence or absence of H_2O_2 (Figure 3) or *tert*-butylhydroperoxide until maximal inactivation was achieved. Mitochondria were then solubilized and treated with glutaredoxin and GSH. In contrast to the thioredoxin reducing system, the glutaredoxin system was capable of fully reactivating KGDH inactivated by treatment of mitochondria with either H_2O_2 (Figure 3) or *tert*-butylhydroperoxide (results not shown). Reactivation was complete within 30 s at all glutaredoxin concentrations tested. GSH had no effect when added in the absence of glutaredoxin. It is well-known that GSH is required for the reactivation of glutaredoxin following deglutathionylation of protein by glutaredoxin. KGDH reactivation was dependent on the addition of GSH only when glutaredoxin concentrations equaled ≤ 0.1 μM. At glutaredoxin concentrations of >0.1 μM, increasing degrees of KGDH reactivation were observed in the absence of added GSH, indicating GSH acts specifically to regenerate catalytically active glutaredoxin. It has previously been shown that glutaredoxin does not catalyze the reduction of protein disulfides (*23*−*25*). In cases where glutaredoxin has been used to reduce protein disulfides (RS−SR), there is an absolute requirement for GSH to first form a mixed disulfide with the protein (RS−SG) followed by subsequent deglutathionylation by glutaredoxin (*23*−*25*). The results shown here coupled with the inability of thioredoxin to restore KGDH activity indicate that H_2O_2 treatment of mitochondria induces reversible glutathionylation of KGDH.

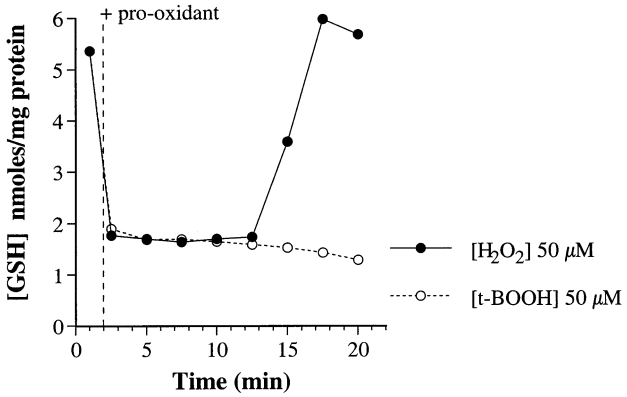

FIGURE 4: Time-dependent alterations in GSH levels upon treatment of mitochondria with H_2O_2 or *tert*-butylhydroperoxide. Intact rat heart mitochondria (0.25 mg/mL) were incubated with 15 mM α-ketoglutarate (0 min) as a respiratory substrate and 2.0 mM ADP (1.0 min) to initiate state 3 respiration. At 2.0 min, 50 μM H_2O_2 or *tert*-butylhydroperoxide was introduced. Mitochondria were solubilized with 0.1% Triton X-100 at the times indicated on the abscissa and diluted into mobile phase, and membranes were removed as described in Materials and Methods. Samples were then resolved by reverse phase HPLC utilizing a C_{18} column and GSH levels determined by electrochemical detection at −0.75 V.

Intramitochondrial Redox Status. In contrast to that with H_2O_2, *tert*-butylhydroperoxide-induced inactivation of KGDH was not reversible under the time frame of our experiments (Figure 1). Additional evidence for glutathionylation as a mechanism for reversible H_2O_2-mediated KGDH inactivation was sought by comparing the response of the glutathione redox status to *tert*-butylhydroperoxide versus H_2O_2 using reverse phase HPLC and electrochemical detection. Intact, respiring mitochondria were incubated in the presence of either 50 μM H_2O_2 or *tert*-butylhydroperoxide. Control mitochondria respired in the absence of peroxide. GSH levels dropped to approximately 30% of control values immediately upon treatment of intact mitochondria with peroxide and subsequently established a new steady state level (Figure 4). GSH levels recovered (Figure 4) at the same time as KGDH activity (Figure 1) in mitochondria treated with H_2O_2. In contrast, GSH levels continued to decline at a very slow rate in *tert*-butylhydroperoxide-treated mitochondria, with no recovery evident during the time course of the experiment (Figure 4). It has previously been shown in brain and liver mitochondria that *tert*-butylhydroperoxide causes a decline in the level of GSH and an increase in the level of GSSG, with recovery of the GSH/GSSG ratio being dependent on experimental conditions (*26*−*30*). Deglutathionylation of protein by glutaredoxin requires reduced glutathione to restore catalytically active glutaredoxin. Thus, these results provide an explanation for the irreversible nature of *tert*-butylhydroperoxide-induced inactivation of KGDH and further support the conclusion that peroxide-induced inactivation is due to glutathionylation.

It is known that there are at least three enzymes that scavenge H_2O_2 within cardiac mitochondria: glutathione peroxidase, peroxiredoxin, and catalase. To assess the removal of H_2O_2 by mitochondria, H_2O_2 concentrations were measured spectrofluorometrically. As shown in Figure 5, approximately 80% of the H_2O_2 was removed within 3 min of addition with a pseudo-first-order rate constant of 0.48 min^{-1} and a $t_{1/2}$ of 1.4 min for 50 μM H_2O_2. Recovery of GSH concentrations and KGDH activity to control values

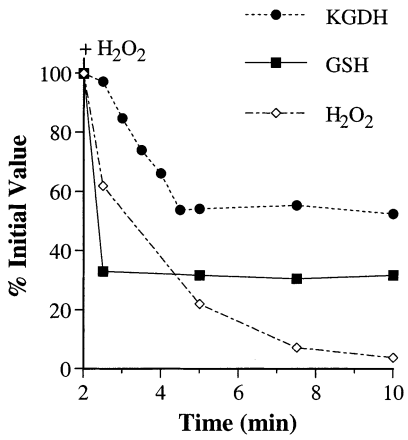

FIGURE 5: Time-dependent alterations in KGDH activity and levels of H_2O_2 and GSH upon treatment of mitochondria with H_2O_2. Incubation of mitochondria with H_2O_2 and measurements of KGDH activity and levels of H_2O_2 and GSH were performed as described in the legends of Figures 1 and 4 and Materials and Methods. KGDH activity was determined at the times indicated on the abscissa, with time intervals between data points during the inactivation process shorter than those depicted in Figure 1. Values for GSH levels were taken from data presented in Figure 4.

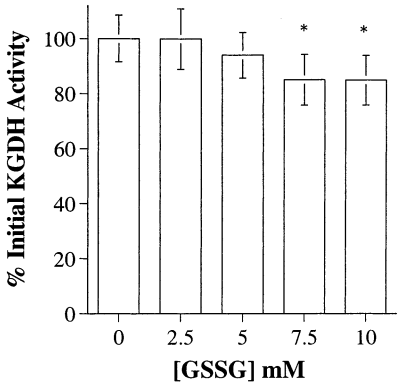

FIGURE 6: Effect of GSSG on KGDH activity. Mitochondria (0.25 mg/mL) were solubilized with 0.1% Triton X-100 and then treated for 5.0 min with GSSG at the concentrations indicated on the abscissa. Samples were subsequently diluted to 0.05 mg/mL, and KGDH activity was assayed spectrophotometrically. Values represent means ($n = 5$) ± SD. *P* values (determined from a two-tailed *t* test) where asterisks indicate $p \leq 0.01$ relative control values.

occurred only upon complete consumption of H_2O_2 (Figures 1 and 4). Under the conditions used in our experiments, respiratory substrate was required for removal of H_2O_2, indicating the requirement for reducing equivalents (results not shown). NADPH is required for the reduction of glutathione peroxidase and peroxiredoxin cofactors. This, in combination with the rapid decline in GSH levels, suggests that the glutathione peroxidase/glutathione reductase system plays a significant role in H_2O_2 clearance under the conditions used in our experiments.

Effects of GSH and GSSG on KGDH Activity. Upon addition of H_2O_2 to intact mitochondria, KGDH activity declines at a steady rate during the subsequent 3 min (Figure 5). This is in contrast to GSH, which exhibits a rapid decline in concentration within the first 30 s (Figures 4 and 5). This suggests that GSSG or a reactive metabolite of GSH is the species involved in the glutathionylation and inactivation of KGDH. Solubilized mitochondria were therefore incubated in the presence of varying concentrations of GSH and GSSG under a variety of conditions to assess potential mechanisms of H_2O_2-mediated inactivation of KGDH. GSH (0−10 mM) had no effect on KGDH activity (results not shown). In contrast, GSSG caused a 20% decline in KGDH activity but only at concentrations of >5.0 mM (Figure 6). Conditions for enhancing this 20% decline were extensively investigated. Neither the presence of H_2O_2 nor any combination of α-ketoglutarate, CoASH, thymine pyrophosphate, NAD^+, NADH, and $MgCl_2$ was able to enhance the degree of inactivation. Additionally, *in vitro* inactivation of KGDH by GSSG could not be reversed by the glutaredoxin/GSH system, indicating that the mechanism of inactivation differs from that which occurs *in situ*. Together, these results indicate that H_2O_2-induced glutathionylation of KGDH is specific and dependent on the distinct composition of mitochondria exposed to oxidative stress.

Inactivation of Purified KGDH by Diamide and GSH. Utilizing a proteomics approach, diamide in combination with glutathione has been used to demonstrate the susceptibility of specific proteins to glutathionylation (*31*). Moreover, an additional advantage of this approach is that sufficient quantities of purified protein can be modified and analyzed. Therefore, if exposure of pure KGDH to diamide and GSH mimicked the mode of KGDH inactivation observed upon treatment of intact mitochondria with H_2O_2, this method would facilitate identification of a specific KGDH subunit(s) and site(s) susceptible to reversible glutathionylation in response to H_2O_2-mediated alterations in mitochondrial redox status. To evaluate the sensitivity of KGDH to *in vitro* glutathionylation, purified KGDH (0.3 mg/mL) was treated with varying concentrations of GSH and 100 μM diamide for 10 min. Increasing concentrations of GSH caused a progressive diamide-dependent decline in KGDH activity to a maximum level of inactivation of 50−60% (Figure 7A). This decline was reversed upon treatment of inactivated enzyme with DTT. In contrast, treatment with glutaredoxin at a concentration 10-fold greater than that required to reverse inactivation observed in intact mitochondria was unable to reactivate diamide/GSH-inactivated KGDH (Figure 7B). This indicates a mode of inactivation distinct from that occurring in intact mitochondria. This is not surprising given that diamide can prime numerous sulfhydryl groups for glutathionylation, many of which may not undergo modification under more physiological conditions of oxidative stress (*31*, *32*). These results underscore the exquisite specificity of processes occurring within intact mitochondria.

DISCUSSION

A unique contribution of this study is evidence that the Krebs cycle enzyme KGDH undergoes reversible glutathionylation in response to alterations in mitochondrial redox status. This process mediates loss and recovery of KGDH activity in intact mitochondria treated with a physiologically relevant oxidant. We have demonstrated that KGDH is highly resistant to direct inactivation by H_2O_2 or *tert*-butylhydroperoxide and that peroxides do not appear to exert their effects through the production of more reactive oxygen radical species. Treatment of inactivated KGDH with DTT resulted in recovery of KGDH activity, demonstrating that inactivation is due, in large part, to the oxidation of key sulfhydryl residues. Thioredoxin failed to reactivate KGDH,

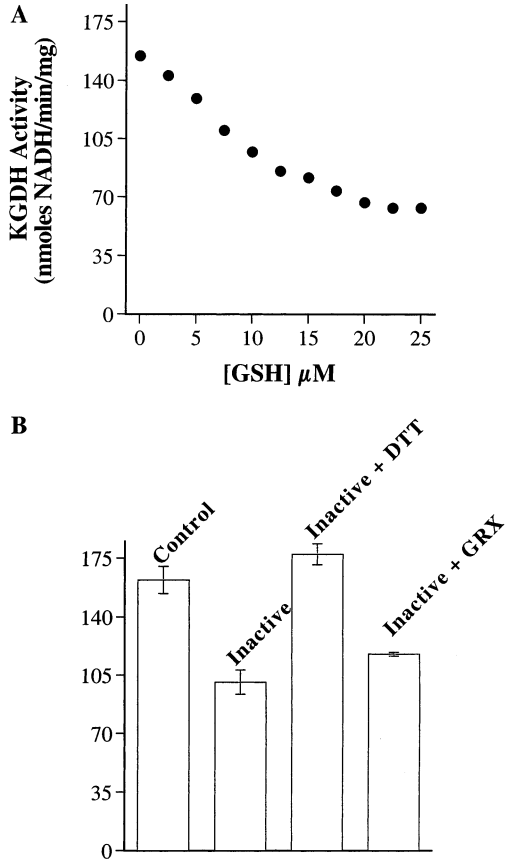

FIGURE 7: Inactivation of purified KGDH by diamide and GSH. (A) Purified KGDH (0.3 mg/mL) was treated with a range of GSH concentrations (from 0 to 25 μM) and 100 μM diamide for 10 min. Samples were then diluted to 0.03 mg/mL, and KGDH activity was assayed as described in Materials and Methods. (B) Purified KGDH (0.3 mg/mL) was either untreated (Control) or treated with 20 μM GSH and 100 μM diamide for 10 min (Inactive). Where indicated, samples were subsequently treated with either 20 mM DTT or 1 unit/mL glutaredoxin for 5 or 2.5 min, respectively, followed by measurement of KGDH activity.

indicating that disulfide formation is not responsible for enzyme inactivation. Importantly, KGDH activity was restored upon addition of glutaredoxin, a thiol disulfide oxidoreductase specific for the removal of glutathione from protein−SSG species. Additionally, glutaredoxin was able to reverse KGDH inactivation in the absence of GSH, indicating that glutaredoxin is not acting to reduce protein disulfide(s) (*23*−*25*). Assessment of the redox status of the mitochondria demonstrated that reduced glutathione levels fall (~70% within 30 s) prior to enzyme inactivation but recover at the same time as enzyme activity. These results support the conclusion that KGDH activity is modulated via H_2O_2-induced glutathionylation and deglutathionylation.

Upon close inspection, it is clear that there is a specific order of events that lends insight into potential mechanisms by which mitochondria assess redox status. After H_2O_2 treatment for 30 s, approximately 40% of the H_2O_2 has been consumed and GSH levels have fallen to approximately 30% of control values, establishing a new steady state concentration. In contrast to the concentration of GSH, KGDH activity continues to decline at a steady rate for approximately 3 min after the addition of H_2O_2. *tert*-Butylhydroperoxide induces a similar time-dependent decline in both GSH levels and KGDH activity. These observations support the notion that KGDH is sensitive to GSSG and/or a reactive metabolite of GSH. Nevertheless, while KGDH can be partially inactivated by GSSG, the mechanism appears to be distinct from that which occurs within intact mitochondria. The maximal level of inactivation of KGDH upon exposure to GSSG is significantly lower than that observed in intact mitochondria, and importantly, addition of glutaredoxin does not result in recovery of enzyme activity. In intact mitochondria, substrate must be present to induce KGDH inactivation. However, GSH levels fall even in the absence of substrate (results not shown). This implies that the enzyme must be catalytically active to be susceptible to H_2O_2-induced inactivation. Nevertheless, addition of various combinations of substrates and cofactors of KGDH and/or H_2O_2 did not enhance GSSG-dependent inactivation. Together, these results suggest that KGDH is not passively inactivated in mitochondria exposed to oxidative stress but that there is a highly specific set of conditions that are necessary to induce inactivation. This process is likely to be dependent on the intrinsic structure of KGDH within the mitochondria, the coordinated response of multiple mitochondrial components, and/or an enzymatic process akin to well-known kinase/phosphatase systems. Interestingly, *tert*-butylhydroperoxide has been shown to induce formation of protein mixed disulfides with glutathione within brain mitochondria; however, the proteins were not identified (*30*). Identification of likely targets of glutathionylation, namely, KGDH, will aid in the elucidation of factors that control this form of post-translational modification.

Available methods lack sufficient sensitivity for detecting glutathionylation of KGDH in mitochondria exposed to a relatively mild oxidative stress. In addition, KGDH does not represent an abundant protein and readily aggregates under nonreducing conditions required for analysis. We therefore sought evidence for a likely site of modification using a variety of *in vitro* systems where sufficient quantities of protein could be analyzed. However, none of the systems tested mimics the selective nature of modification and the rapid reversal of inactivation by glutaredoxin observed when intact mitochondria are treated with H_2O_2. Utilizing a proteomics approach, numerous proteins have been identified which undergo glutathionylation upon treatment of cells with the strong oxidant diamide in combination with GSH (*31*). A large number of these proteins do not undergo detectable modification when diamide is replaced with H_2O_2 (*31*). Taken together, these findings underscore two important points. (1) The use of diamide and GSH is an efficient means for identifying potential candidates for glutathionylation; however, the physiological significance of such results must be interpreted with caution. (2) Methods for detecting glutathionylation under mild oxidative stress must be improved to identify low-abundance proteins and/or those with properties unfavorable for analysis.

While the exact mechanism by which KGDH becomes glutathionylated remains to be defined, it is likely that reactivation is an enzymatic process. Treatment of inactive KGDH with glutaredoxin facilitated recovery of enzyme activity. Glutaredoxin is characterized as a specific and efficient catalyst of protein deglutathionylation and requires reduced glutathione to maintain catalytic activity. Consistent with this requirement, KGDH activity recovered in H_2O_2-treated mitochondria in parallel with GSH levels, while *tert*-butylhydroperoxide-treated mitochondria exhibited no re-

covery in GSH concentration or KGDH activity. Recently, a novel glutaredoxin (GRx2) containing a mitochondrial leader sequence was identified in human and mouse tissue (*33*, *34*). Unlike other glutaredoxin isoforms, GRx2 is relatively insensitive to oxidative inactivation, making it an effective enzyme for an oxidatively dynamic environment like the mitochondria (*34*). The glutaredoxin used to reactivate KGDH in this study was the cytosolic form of the enzyme (GRx1). The concentration of GRx1 necessary to completely reverse enzyme inactivation resulting from H_2O_2 was small. This is in stark contrast to the high concentration of DTT necessary to recover KGDH activity under the same conditions. Due to the short period of time and the relatively low concentration of glutaredoxin necessary for reactivation in our model system, if the glutaredoxin isoform within the mitochondria exhibits similar activity it is likely that this enzyme is responsible for reactivation of KGDH in intact mitochondria.

The mechanisms responsible for enzyme inactivation and reactivation appear to be tightly linked to oxidant scavenging mechanisms, thus facilitating a coordinated response to changes in the mitochondrial redox status. There are three enzymes present within cardiac mitochondria that can convert H_2O_2 to O_2 and H_2O: catalase (*35*, *36*), peroxiredoxin (*37*), and glutathione peroxidase. Glutathione peroxidase carries out this reaction at the expense of reduced glutathione. Glutathione reductase regenerates the reduced glutathione pool upon oxidation of NADPH provided by isocitrate dehydrogenase, malic enzyme, nicotinamide nucleotide transhydrogenase, and NAD^+ and NADH kinase (*38*−*42*). Upon incubation of intact mitochondria with H_2O_2, there is a profound and immediate decline in reduced glutathione levels. Furthermore, mitochondria must have substrate to scavenge H_2O_2, suggesting the requirement for reducing equivalents. These data suggest that glutathione peroxidase and glutathione reductase are responsible, at least in part, for removing H_2O_2 under the conditions of our experiments. Peroxiredoxin may also contribute to the antioxidant response given that this enzyme requires NADPH for catalytic activity. A recent report indicates that glutathione peroxidase is largely responsible for H_2O_2 detoxification in cardiac mitochondria (*43*). Catalase has been suggested to rescue mitochondria from high concentrations of H_2O_2 (*36*). Our data indirectly support these notions. Importantly, the fact that glutathione may be critical for both the antioxidant response and KGDH inactivation and/or reactivation indicates a coordinated response of mitochondria to oxidative stress.

To maintain mitochondrial and cellular viability, the mitochondria must respond to a dynamic redox environment. Initially, oxidants induce a decrease in GSH levels. We have provided evidence indicating that KGDH, a key Krebs cycle enzyme, is then glutathionylated. Finally, glutathionylation is reversed upon recovery of the GSH through an enzymatic process that is likely dependent on glutaredoxin. Therefore, glutathionylation of KGDH may represent a suitable protective response. KGDH contains several sulfhydryl residues that are vital to catalysis (*20*, *21*). It has been suggested that glutathionylation may protect sulfhydryl residues from irreversible oxidative damage (*8*, *24*, *32*). Additionally, by limiting the activity of an NADH-producing enzyme, Tretter and Adam-Vizi (*44*) found it is possible to limit the amount of reducing equivalents available for electron transport, the ultimate source of oxygen radicals. In support of this possibility, KGDH activity and GSH concentration do not vary under relatively mild oxidative stress resulting from elevated H_2O_2 production during ADP-independent (state 4) relative to ADP-dependent (state 3) mitochondrial respiration (not shown) (*9*, *10*, *45*−*47*). Thus, inhibition of KGDH activity by glutathionylation would likely occur under conditions where oxidant production exceeds antioxidant capacity and the mitochondrial glutathione content declines. It has previously been reported that, in *in vivo* dog models of cardiac ischemia/reperfusion, the glutathione content of cardiac mitochondria isolated from control versus reperfused tissue drops from 2.6 to 1.4 nmol/mg (*48*) and from 3.7 to 2.2 nmol/mg (*49*). Interestingly, KGDH activity has been reported to decline during cardiac ischemia/reperfusion, although the mechanism by which this occurs has not been fully elucidated (*13*, *50*). There are numerous pathophysiologic conditions during which redox status and, in particular, the GSH/GSSG ratio are perturbed (*48*, *49*, *51*, *52*). Identification of enzymes that are responsive to these alterations and consideration of conditions that evoke changes in redox status will provide an understanding of the physiological significance of the metabolic response.

REFERENCES

1. Kamata, H., and Hirata, H. (1999) Redox regulation of cellular signalling, *Cell. Signalling 11*, 1−14.
2. Aslund, F., and Beckwith, J. (1999) Bridge over troubled waters: sensing stress by disulfide bond formation, *Cell 96*, 751−753.
3. Klatt, P., and Lamas, S. (2000) Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress, *Eur. J. Biochem. 267*, 4928−4944.
4. Rhee, S. G. (1999) Redox signaling: hydrogen peroxide as intracellular messenger, *Exp. Mol. Med. 31*, 53−59.
5. Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997) Oxidants as stimulators of signal transduction, *Free Radical Biol. Med. 22*, 269−285.
6. Finkel, T. (2000) Redox-dependent signal transduction, *FEBS Lett. 476*, 52−54.
7. Gabbita, S. P., Robinson, K. A., Stewart, C. A., Floyd, R. A., and Hensley, K. (2000) Redox regulatory mechanisms of cellular signal transduction, *Arch. Biochem. Biophys. 376*, 1−13.
8. Thomas, J. A., Poland, B., and Honzatko, R. (1995) Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation, *Arch. Biochem. Biophys. 319*, 1−9.
9. Chance, B., and Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem. 17*, 65−134.
10. Cadenas, E., and Davies, K. J. (2000) Mitochondrial free radical generation, oxidative stress, and aging, *Free Radical Biol. Med. 29*, 222−230.
11. Nulton-Persson, A. C., and Szweda, L. I. (2001) Modulation of mitochondrial function by hydrogen peroxide, *J. Biol. Chem. 276*, 23357−23361.
12. Berlett, B. S., and Stadtman, E. R. (1997) Protein oxidation in aging, disease, and oxidative stress, *J. Biol. Chem. 272*, 20313−20316.
13. Lucas, D. T., and Szweda, L. I. (1999) Declines in mitochondrial respiration during cardiac reperfusion: age-dependent inactivation of α-ketoglutarate dehydrogenase, *Proc. Natl. Acad. Sci. U.S.A. 96*, 6689−6693.
14. Beal, M. F. (1995) Aging, energy, and oxidative stress in neurodegenerative diseases, *Ann. Neurol. 38*, 357−366.
15. Kish, S. J. (1997) Brain energy metabolizing enzymes in Alzheimer's disease: α-ketoglutarate dehydrogenase complex and cytochrome oxidase, *Ann. N.Y. Acad. Sci. 826*, 218−228.
16. Mastrogiacomo, F., Bergeron, C., and Kish, S. J. (1993) Brain α-ketoglutarate dehydrogenase complex activity in Alzheimer's disease, *J. Neurochem. 61*, 2007−2014.
17. Mizuno, Y., Ikebe, S., Hattori, N., Nakagawa-Hattori, Y., Mochizuki, H., Tanaka, M., and Ozawa, T. (1995) Role of mito-

chondria in the etiology and pathogenesis of Parkinson's disease, *Biochim. Biophys. Acta 1271*, 265−274.
18. Chrestensen, C. A., Starke, D. W., and Mieyal, J. J. (2000) Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis, *J. Biol. Chem. 275*, 26556−26565.
19. Han, D., Antunes, F., Daneri, F., and Cadenas, E. (2002) Mitochondrial superoxide anion production and release into intermembrane space, *Methods Enzymol. 249*, 271.
20. Perham, R. N. (1991) Domains, motifs, and linkers in 2-oxo acid dehydrogenase multienzyme complexes: a paradigm in the design of a multifunctional protein, *Biochemistry 30*, 8501−8512.
21. Sheu, K. F., and Blass, J. P. (1999) The α-ketoglutarate dehydrogenase complex, *Ann. N.Y. Acad. Sci. 893*, 61−78.
22. Eaton, P., Byers, H. L., Leeds, N., Ward, M. A., and Shattock, M. J. (2002) Detection, quantitation, purification, and identification of cardiac proteins S-thiolated during ischemia and reperfusion, *J. Biol. Chem. 277*, 9806−9811.
23. Gravina, S. A., and Mieyal, J. J. (1993) Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase, *Biochemistry 32*, 3368−3376.
24. Mieyal, J. J., Gravina, S. A., Mieyal, P. A., Srinivasan, U., and Starke, D. W. (1995) Glutathionyl specificity of thioltransferases: Mechanistic and physiological implications, in *Biothiols in health and disease* (Packer, L., and Cadenas, E., Eds.) pp 305−372, Marcel Dekker, New York.
25. Jung, C. H., and Thomas, J. A. (1996) S-Glutathiolated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione, *Arch. Biochem. Biophys*. *335*, 61−72.
26. Rokutan, K., Kawai, K., and Asada, K. (1987) Inactivation of 2-oxoglutarate dehydrogenase in rat liver mitochondria by its substrate and *tert*-butyl hydroperoxide, *J. Biochem. 101*, 415−422.
27. Brodie, A. E., and Reed, D. J. (1992) Glutathione disulfide reduction in tumor mitochondria after *tert*-butyl hydroperoxide treatment, *Chem.-Biol. Interact. 84*, 125−132.
28. Liu, H., and Kehrer, J. P. (1996) The reduction of glutathione disulfide produced by *tert*-butyl hydroperoxide in respiring mitochondria, *Free Radical Biol. Med. 20*, 433−442.
29. Olafsdottir, K., and Reed, D. J. (1988) Retention of oxidized glutathione by isolated rat liver mitochondria during hydroperoxide treatment, *Biochim. Biophys. Acta 964*, 377−382.
30. Ravindranath, V., and Reed, D. J. (1990) Glutathione depletion and formation of glutathione-protein mixed disulfide following exposure of brain mitochondria to oxidative stress, *Biochem. Biophys. Res. Commun. 169*, 1075−1079.
31. Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., Vandekerckhove, J., Gianazza, E., and Ghezzi, P. (2002) Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes, *Proc. Natl. Acad. Sci. U.S.A. 99*, 3505−3510.
32. Barrett, W. C., DeGnore, J. P., Konig, S., Fales, H. M., Keng, Y. F., Zhang, Z. Y., Yim, M. B., and Chock, P. B. (1999) Regulation of PTP1B via glutathionylation of the active site cysteine 215, *Biochemistry 38*, 6699−6705.
33. Gladyshev, V. N., Liu, A., Novoselov, S. V., Krysan, K., Sun, Q. A., Kryukov, V. M., Kryukov, G. V., and Lou, M. F. (2001) Identification and characterization of a new mammalian glutaredoxin (thioltransferase), Grx2, *J. Biol. Chem. 276*, 30374−30380.
34. Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M., and Holmgren, A. (2001) Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms, *J. Biol. Chem. 276*, 26269−26275.
35. Nohl, H., and Hegner, D. (1978) Evidence for the existence of catalase in the matrix space of rat-heart mitochondria, *FEBS Lett. 89*, 126−130.
36. Radi, R., Turrens, J. F., Chang, L. Y., Bush, K. M., Crapo, J. D., and Freeman, B. A. (1991) Detection of catalase in rat heart mitochondria, *J. Biol. Chem. 266*, 22028−22034.
37. Seo, M. S., Kang, S. W., Kim, K., Baines, I. C., Lee, T. H., and Rhee, S. G. (2000) Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate, *J. Biol. Chem. 275*, 20346−20354.
38. Jo, S. H., Son, M. K., Koh, H. J., Lee, S. M., Song, I. H., Kim, Y. O., Lee, Y. S., Jeong, K. S., Kim, W. B., Park, J. W., Song, B. J., Huh, T. L., and Huhe, T. L. (2001) Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase, *J. Biol. Chem. 276*, 16168−16176.
39. Vogel, R., Wiesinger, H., Hamprecht, B., and Dringen, R. (1999) The regeneration of reduced glutathione in rat forebrain mitochondria identifies metabolic pathways providing the NADPH required, *Neurosci. Lett. 275*, 97−100.
40. McGuinness, E. T., and Butler, J. R. (1985) NAD^+ kinase−a review, *Int. J. Biochem. 17*, 1−11.
41. Iwahashi, Y., Hitoshio, A., Tajima, N., and Nakamura, T. (1989) Characterization of NADH kinase from *Saccharomyces cerevisiae*, *J. Biochem. 105*, 588−593.
42. Iwahashi, Y., and Nakamura, T. (1989) Localization of the NADH kinase in the inner membrane of yeast mitochondria, *J. Biochem. 105*, 916−921.
43. Antunes, F., Han, D., and Cadenas, E. (2002) Relative contributions of heart mitochondria glutathione peroxidase and catalase to H_2O_2 detoxification in in vivo conditions, *Free Radical Biol. Med. 33*, 1260−1267.
44. Tretter, L., and Adam-Vizi, V. (2000) Inhibition of Krebs cycle enzymes by hydrogen peroxide: A key role of α-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress, *J. Neurosci. 20*, 8972−8979.
45. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef-heart mitochondria, *Arch. Biochem. Biophys. 180*, 248−257.
46. Cadenas, E., and Boveris, A. (1980) Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria, *Biochem. J. 188*, 31−37.
47. Turrens, J. F., and Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria, *Biochem. J. 191*, 421−427.
48. Kajiyama, K., Pauly, D. F., Hughes, H., Yoon, S. B., Entman, M. L., and McMillin-Wood, J. B. (1987) Protection by verapamil of mitochondrial glutathione equilibrium and phospholipid changes during reperfusion of ischemic canine myocardium, *Circ. Res. 61*, 301−310.
49. Nishinaka, Y., Sugiyama, S., Yokota, M., Saito, H., and Ozawa, T. (1993) Protective effect of FK506 on ischemia/reperfusion-induced myocardial damage in canine heart, *J. Cardiovasc. Pharmacol. 21*, 448−454.
50. Sadek, H. A., Humphries, K. M., Szweda, P. A., and Szweda, L. I. (2002) Selective inactivation of redox-sensitive mitochondrial enzymes during cardiac reperfusion, *Arch. Biochem. Biophys. 406*, 222−228.
51. Cock, H. R., Tong, X., Hargreaves, I. P., Heales, S. J., Clark, J. B., Patsalos, P. N., Thom, M., Groves, M., Schapira, A. H., Shorvon, S. D., and Walker, M. C. (2002) Mitochondrial dysfunction associated with neuronal death following status epilepticus in rat, *Epilepsy Res. 48*, 157−168.
52. Bharath, S., Hsu, M., Kaur, D., Rajagopalan, S., and Andersen, J. K. (2002) Glutathione, iron and Parkinson's disease, *Biochem. Pharmacol. 64*, 1037−1048.

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