# Reversible Inhibition of α-Ketoglutarate Dehydrogenase by Hydrogen Peroxide: Glutathionylation and Protection of Lipoic Acid†

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ABSTRACT: We have previously demonstrated that when cardiac mitochondria were challenged with $H_2O_2$, NADH production and oxidative phosphorylation declined. Upon consumption of $H_2O_2$, mitochondrial function was restored. These alterations were due, in large part, to reversible glutathionylation and inhibition of the Krebs cycle enzyme α-ketoglutarate dehydrogenase. The current study was undertaken to identify the site and consequences of α-ketoglutarate dehydrogenase glutathionylation. Mitochondria were treated with $H_2O_2$ for varying periods of time. Protein sulfhydryls that had undergone $H_2O_2$ mediated glutathionylation were specifically derivatized with *N*-ethylmaleimide-biotin. Subsequent purification of biotin labeled (glutathionylated) protein and Western blot analysis revealed that the E2 subunit of α-ketoglutarate dehydrogenase was reversibly glutathionylated. Further analysis revealed that lipoic acid, a required cofactor covalently attached to the E2 subunit, was the site of glutathionylation. The relative level of glutathionylated lipoic acid closely paralleled the degree of enzyme inhibition and reactivation. Glutathionylation of α-ketoglutarate dehydrogenase protected lipoic acid from modification by the electrophilic lipid peroxidation product 4-hydroxy-2-nonenal. Glutathionylation of α-ketoglutarate dehydrogenase can therefore be viewed as an antioxidant response protecting the enzyme from oxidative damage.

Glutathione (GSH[1]) is a cysteine containing tripeptide (γ-L-glutamyl-L-cysteinylglycine) present at millimolar concentrations within cells and organelles (*1*, *2*). As a required cofactor for the reductive elimination of hydroperoxides by glutathione peroxidase, glutathione is rapidly oxidized resulting in the formation of a disulfide between two glutathione molecules. Glutathione reductase then catalyzes the conversion of oxidized glutathione (GSSG) to GSH maintaining a reductive environment (*1*, *2*). Glutathione has more recently been recognized as a regulatory molecule (*3*−*6*). Mixed disulfides between protein sulfhydryl groups and GSH can form via nonenzymatic and potentially enzymatic mechanisms (*3*−*6*). Protein glutathionylation is readily reversible through the action of glutaredoxin, an enzyme that regenerates reduced sulfhydryls on protein cysteine residues with the concomitant production of GSSG (*3*, *7*−*12*). Given the reversible nature of this modification and the importance of certain sulfhydryl groups for protein function, protein glutathionylation and deglutathionylation may be viewed as the redox equivalent of protein phosphorylation and dephosphorylation (*4*, *6*).

We have previously reported that when cardiac mitochondria are challenged with hydrogen peroxide ($H_2O_2$), NADH-linked ADP-dependent respiratory activity is diminished (*13*). Maximal rates of respiration are reestablished upon elimination of $H_2O_2$ by the mitochondria. Analysis of specific components of the electron transport chain and Krebs cycle revealed that $H_2O_2$ exerted these effects by reversibly inhibiting α-ketoglutarate dehydrogenase (KGDH) (*13*, *14*), a critical regulatory enzyme that limits the rate of NADH production and oxidative phosphorylation (*15*−*19*). Glutaredoxin but not the thioredoxin/thioredoxin reductase system was capable of fully reactivating KGDH (*14*). It was concluded that the rates of mitochondrial respiration and oxidative phosphorylation could be modulated by $H_2O_2$ through the reversible glutathionylation of KGDH (*14*). This may represent a regulated antioxidant response, protecting key sulfhydryl groups on KGDH from irreversible oxidative damage and diminishing supply of NADH to the electron transport chain, a known source of $H_2O_2$ (*20*−*24*).

KGDH is a multienzyme complex that consists of multiple copies of three subunits: E1 (α-ketoacid decarboxylase); E2 (dihydrolipoyl transacetylase); and E3 (dihydrolipoamide dehydrogenase) (*25*). Two potential sites of glutathionylation that would directly impact enzymatic activity are evident. The E2 subunit contains a lipoic acid covalently bound to a lysine residue. Lipoic acid cycles between sulfhydryl (reduced) and disulfide (oxidized) states, providing for the

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[1] Abbreviations: KGDH, α-ketoglutarate dehydrogenase; E1, α-ketoacid decarboxylase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoamide dehydrogenase; $H_2O_2$, hydrogen peroxide; GRx, glutaredoxin; HNE, 4-hydroxy-2-nonenal; GSH, reduced glutathione; GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; biotin-NEM, biotin-*N*-ethylmaleimide; biotin-HPDP, *N*-[-6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide; NAC, *N*-acetylcysteine; MOPS, 3-(*N*-morpholino) propanesulfonic acid.

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reduction of a disulfide on the enzyme's E3 subunit. These processes are required for the conversion of $NAD^+$ to NADH (*25*). Glutathionylation of any one of these sulfhydryl functionalities would therefore be expected to inhibit protein function. The current study was undertaken to (1) identify the subunit and precise site of glutathionylation on KGDH and (2) assess the potential relevance of $H_2O_2$ mediated KGDH glutathionylation. Mechanisms and consequences of KGDH glutathionylation are presented that provide valuable information on a manner in which protein function can be regulated and protected in response to alterations in redox status.

## MATERIALS AND METHODS

*Isolation of Mitochondria from Rat Heart.* Male Sprague−Dawley rats (250−300 g) obtained from Harlan Laboratories were anesthetized with a mixture of xylazine, ketamine·HCl, and acepromazine (3:3:1) (0.8−1.0 mL/kg). Following midline thoracotomy and pericardiectomy, hearts were excised and perfused with 10 mL ice-cold homogenization buffer (210 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, 5.0 mM MOPS, pH 7.4) to remove blood. Hearts (0.9−1.1 g) were then minced and homogenized in 20 mL of homogenization buffer with a Polytron homogenizer (low setting, 3 s). The homogenate was centrifuged at 500*g* for 5 min (5 °C), and the supernatant was filtered through cheese cloth. The mitochondrial pellet was obtained upon centrifugation of the supernatant at 10000*g* for 10 min (5 °C). After two rinses with ice-cold homogenization buffer, the mitochondria were resuspended into homogenization buffer to a final concentration of 25.0 mg/mL. Protein determinations were made using the bicinchroninic acid (BCA) method (Pierce), using BSA as a standard.

*Incubation of Mitochondria with $H_2O_2$ and/or 4-Hydroxy-2-nonenal.* Mitochondria were diluted to 0.5 mg/mL in buffer composed of 210 mM mannitol, 70 mM sucrose, 10 mM MOPS, and 5.0 mM $K_2HPO_4$ at pH 7.4. Respiration was initiated by the addition of 5.0 mM α-ketoglutarate and allowed to proceed for 2.0 min. $H_2O_2$ (25 $\mu$M) was then added. At indicated times, Triton X-100 was added to a final concentration of 0.05% to prevent further inhibition or reactivation of KGDH (*13*, *14*). For experiments in which mitochondria were treated with 4-hydroxy-2-nonenal (HNE), mitochondria were allowed to respire (5.0 mM α-ketoglutarate) for 2.0 min followed by addition of $H_2O_2$ (0 or 100 $\mu$M). After 2.0 min, HNE (0 or 100 $\mu$M) was added and incubations were allowed to proceed for indicated periods of time. Unreacted HNE was scavenged upon addition of *N*-acetylcysteine (10 mM) and samples were reduced with $NaBH_4$ for subsequent Western blot analysis. Incubations were performed at room temperature.

*Assay for α-Ketoglutarate Dehydrogenase Activity.* Mitochondria were diluted to 0.05 mg/mL in 25 mM MOPS, 0.05% Triton X-100, pH 7.4. KGDH activity was assayed spectrophotometrically as the rate of $NAD^+$ reduction to NADH (340 nm, $\epsilon = 6.2\ M^{-1}$) upon addition of 5.0 mM $MgCl_2$, 2.5 mM α-ketoglutarate, 0.1 mM CoASH, 0.2 mM thiamine pyrophosphate, and 1.0 mM $NAD^+$. Where indicated, glutaredoxin (1.0 U/mL, CalBiochem) and GSH (0.5 mM) were added to deglutathionylate KGDH prior to enzyme analysis.

*Derivitization of Protein with N-Ethylmaleimide and Biotin.* Following treatment of mitochondria with $H_2O_2$ for varying periods of time, Triton X-100 (0.05%) was added. The membrane fraction was then sedimented upon centrifugation for 5.0 min at 20000*g*. Supernatant containing KGDH was treated with 0.5 mM *N*-ethylmaleimide (NEM) for 2.0 min, to derivatize free sulfhydryl residues, followed by addition of *N*-acetylcysteine (0.6 mM) to scavenge unreacted NEM. For biotinylation of sulfhydryl residues that had undergone glutathionylation, glutaredoxin (1.0 unit/mL) and GSH (0.5 mM) were added after NEM treatment. Following deglutathionylation (2.0 min), exposed sulfhydryl groups were biotinylated upon incubation with 1.5 mM Biotin-NEM or the reducible probe *N*-[-6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (Biotin-HPDP, Pierce) for 2.0 min. *N*-Acetylcysteine (1.8 mM) was added to scavenge unreacted Biotin-NEM or Biotin-HPDP. Samples were then subjected to gel filtration chromatography (PD-10 column, Amersham) with 150 mM NaCl, 100 mM $Na_2HPO_4$, 1.0% NP40, pH 7.4 to separate protein from biotin-*N*-acetylcysteine conjugates.

*Purification of Biotinylated Protein.* Following biotinylation and gel filtration chromatography as described above, samples were diluted 1:1 into 2X gel electrophoresis sample buffer (54 mM Tris HCl, 70.5 mM Tris base, 1.0% SDS, 5.0% sucrose, 0.25 mM EDTA, 0.11 mM SERVA Blue G250, and 0.09 mM phenol red at pH 8.5). Samples (500 $\mu$L, 0.1 mg/mL protein) were then mixed with 100 $\mu$L of high affinity streptavidin agarose beads that had been washed with 150 mM NaCl, 100 mM $Na_2HPO_4$, 1.0% NP40, pH 7.4. Following 30 min of incubation, the solution was centrifuged at 750*g* for 1.0 min to pellet the streptavidin agarose beads. Supernatant was collected and 100 mM DTT was added prior to Western blot analysis of the level(s) of protein that did not bind to streptavidin. To identify protein(s) that bound to the streptavidin agarose beads (i.e., biotin labeling corresponds to glutathionylated protein), the pellet was washed once with 1.0 mL of 150 mM NaCl, 100 mM $Na_2HPO_4$, 1.0% NP40, pH 7.4. The conjugate formed between Biotin-HPDP, and protein was then reductively cleaved and protein dissociated from the streptavidin agarose beads upon addition of 1X gel electrophoresis buffer containing 100 mM DTT. Following centrifugation for 1.0 min at 750*g*, supernatant was collected for Western blot analysis.

*Western Blot Analysis.* Samples were suspended in 27 mM Tris HCl, 35.2 mM Tris base, 0.5% SDS, 2.5% sucrose, 0.12 mM EDTA, 0.55 mM SERVA Blue G250, and 0.05 mM phenol red at pH 8.5 and incubated for 10 min at 70 °C. Protein was then resolved on a 10% SDS−PAGE gel and electrotransferred onto nitrocellulose membrane (BioRad). Membrane-immobilized proteins were analyzed utilizing polyclonal antibodies specific to the E1, E2, or E3 subunit of KGDH, lipoic acid, or lipoic acid-HNE as specified. Primary antibody binding was visualized utilizing peroxidase-conjugated secondary antibody and chemiluminescent substrate (Supersignal West Pico from Pierce). Biotin labeled protein was visualized using peroxidase-conjugated avidin (Pierce) and chemiluminescent substrate.

Table 1: Relative Susceptibility of Glutathionylated KGDH to NEM Inactivation[a]

| | 25 $\mu$M $H_2O_2$ | | |
|---|---|---|---|
| | 0 min | 5 min | 15 min |
| no treatment | 100.0 ± 2.1* | 55.3 ± 7.8* | 94.7 ± 6.2 |
| Grx + GSH | 105.8 ± 5.6 | 97.4 ± 9.8 | 101.8 ± 4.2 |
| NEM | 6.4 ± 7.0 | 7.9 ± 6.3 | 7.1 ± 6.3 |
| NEM → Grx + GSH | 5.7 ± 1.2† | 49.5 ± 9.5† | 6.0 ± 1.8 |
| NEM → Grx + GSH → NEM | 4.6 ± 3.8 | 5.8 ± 3.8 | 1.8 ± 1.7 |

[a] Isolated cardiac mitochondria (0.5 mg/mL) were treated with $H_2O_2$ (25 $\mu$M) in the presence of 5.0 mM α-ketoglutarate as respiratory substrate. At 0, 5, and 15 min, mitochondria were solubilized with 0.05% Triton X-100 to prevent further inhibition or reactivation of KGDH. As indicated, samples were then subjected to (1) no treatment; (2) glutaredoxin (1.0 U/mL) and GSH (0.5 mM) for 2.0 min; (3) NEM (0.5 mM) for 2.0 min followed by *N*-acetylcysteine (NAC, 0.6 mM) for 1.0 min; (4) NEM (0.5 mM) for 2.0 min followed by NAC (0.6 mM) for 1.0 min and then glutaredoxin (1.0 U/mL) and GSH (0.5 mM) for 2.0 min; or (5) NEM (0.5 mM) for 2.0 min followed by NAC (0.6 mM) for 1.0 min and then glutaredoxin (1.0 U/mL) and GSH (0.5 mM) for 2.0 min and finally NEM (1.5 mM) for 2.0 min followed by NAC (1.8 mM) for 1.0 min. KGDH activity was then determined and is expressed relative to values obtained from untreated mitochondria with an assigned a value of 100. Values are represented as mean ± standard deviation ($n = 4$). $p$ values (determined from paired $t$ test) where like symbols indicate values compared: * < 0.0001; † < 0.001.

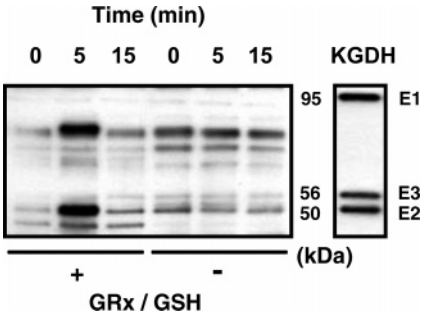



FIGURE 1: Reversible glutathionylation of mitochondrial protein in response to $H_2O_2$. Isolated cardiac mitochondria (0.5 mg/mL) were treated with $H_2O_2$ (25 $\mu$M) in the presence of 5.0 mM α-ketoglutarate as respiratory substrate. At 0, 5, and 15 min, mitochondria were solubilized with 0.05% Triton X-100 and treated with *N*-ethylmaleimide (NEM, 0.5 mM) for 2.0 min to derivatize reduced sulfhydryl groups on protein. Unreacted NEM was scavenged with *N*-acteylcysteine (NAC, 0.6 mM) and samples were incubated with or without glutaredoxin (1.0 U/mL) in the presence of GSH (0.5 mM) to regenerate sulfhydryls that had undergone glutathionylation. Following treatment with biotin-NEM (1.5 mM) for 2.0 min to label exposed protein sulfhydryl groups, unreacted biotin-NEM was scavenged with NAC (1.8 mM). Mitochondrial proteins were then resolved by gel electrophoresis and transferred to nitrocellulose membrane. Glutathionylated (biotinylated) proteins were visualized utilizing HRP-conjugated avidin and chemiluminescence detection. Subunits of KGDH were detected using antibodies specific to the E1, E2, and E3 subunits.

## RESULTS

Isolated rat heart mitochondria (0.5 mg/mL) respiring on α-ketoglutarate (5.0 mM) were treated with 25 $\mu$M $H_2O_2$. At specified times, mitochondrial integrity was disrupted (0.05% Triton X-100) which prevents further inhibition or subsequent reactivation of α-ketoglutarate dehydrogenase (KGDH) (*13*, *14*). Analysis of KGDH activity revealed that the enzyme was maximally inhibited (∼45%) 5.0 min after the addition of $H_2O_2$ and that reactivation was complete at 15 min. As previously reported (*14*), treatment of solubilized mitochondria with glutaredoxin (1.0 unit/mL) and GSH (500 $\mu$M) resulted in full reactivation of $H_2O_2$ inhibited KGDH ($t = 5.0$ min, Table 1). Experiments were performed to determine whether glutathionylation blocks inactivation of KGDH by the sulfhydryl reactive compound *N*-ethylmaleimide (NEM). Following incubation of mitochondria with $H_2O_2$, addition of NEM resulted in inactivation of KGDH independent of duration of exposure to $H_2O_2$ (Table 1). Subsequent treatment with glutaredoxin and GSH resulted in reactivation of enzyme that had been glutathionylated in response to treatment with $H_2O_2$ for 5.0 min. The relative degree of enzyme reactivation corresponded to the level of $H_2O_2$-induced enzyme inhibition. Glutathionylation therefore blocks the enzyme from NEM modification. Finally, following deglutathionylation, the enzyme was susceptible to near complete inactivation by NEM (Table 1). These characteristics were utilized to probe for the subunit and site of glutathionylation.

The fully assembled KGDH enzyme complex consists of multiple copies of three subunits: E1 (α-ketoacid decarboxylase); E2 (dihydrolipoyl transacetylase); and E3 (dihydrolipoamide dehydrogenase) (*25*). To identify the subunit that is glutathionylated in response to $H_2O_2$, mitochondria were incubated with $H_2O_2$ for 0, 5, and 15 min followed by derivatization of free protein sulfhydryl groups with NEM. Protein extracts were then treated with glutaredoxin and GSH to expose glutathionylated sulfhydryl groups. Deglutathionylated sulfhydryl groups were subsequently reacted with NEM-biotin. In this manner, proteins glutathionylated in response to $H_2O_2$ are labeled with biotin. As shown in Figure 1, $H_2O_2$ induced an increase in the level of glutathionylated (biotin-labeled) mitochondrial protein with two major bands evident. This was followed by the disappearance of glutathionylated protein coincident with reactivation of KGDH. Biotinylation required prior treatment with glutaredoxin and GSH providing evidence for the specificity of the labeling procedure (Figure 1). One of the biotin labeled bands had electrophoretic mobility consistent with the molecular weight of the E2 subunit of KGDH (∼50 kDa). Biotin labeled proteins were not evident at molecular weights corresponding to the E1 or E3 subunits of KGDH (Figure 1). While other glutathionylated proteins are present (Figure 1), because of our interest in KGDH subsequent experiments focused on the 50 kDa E2 subunit.

To gain more direct evidence for glutathionylation of the E2 subunit, biotin-labeled mitochondrial proteins were bound to agarose-immobilized streptavidin. No avidin-HPR binding was evident in the supernatant indicating complete depletion of biotinylated protein (Figure 2A). Protein that did not bind to streptavidin was subjected to Western blot analysis using an antibody to the E2 subunit of KGDH. As shown in Figure 2A, loss of the E2 subunit was evident in samples that had been subjected to $H_2O_2$ treatment for 5.0 min. Therefore the E2 subunit of KGDH appears to be biotinylated exclusively under conditions where the enzyme was glutathionylated and inhibited in response to $H_2O_2$ (Figure 2A). The binding affinity between biotin and streptavidin is strong as evidenced by interactions in SDS sample buffer which was used to minimize nonspecific interactions (Figure 2A). In an effort to provide further evidence that the E2 subunit is glutathionylated in response to $H_2O_2$, samples were labeled with biotin using a reagent (Biotin-HPDP) that forms disulfides with

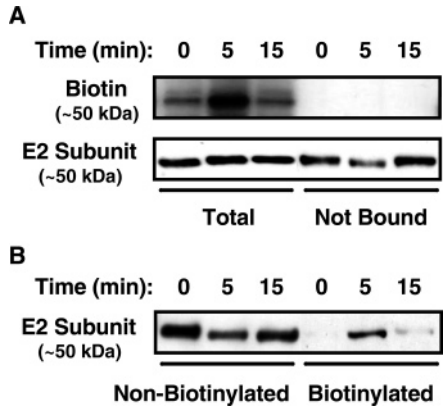



FIGURE 2: Identification of the E2 subunit of KGDH as a site of $H_2O_2$-induced glutathionylation. A. Isolated cardiac mitochondria (0.5 mg/mL) were treated with $H_2O_2$ (25 $\mu$M) in the presence of 5.0 mM α-ketoglutarate as respiratory substrate. At 0, 5, and 15 min, mitochondria were solubilized with 0.05% Triton X-100 and free and glutathionylated protein sulfhydryls were derivatized with NEM and NEM-biotin, respectively, as described in the legend to Figure 1 and in Materials and Methods. After labeling proteins with NEM-biotin, gel filtration chromatography was performed to remove low molecular weight biotin conjugates. Following dilution into SDS gel electrophoresis sample buffer, samples were incubated (30 min) with avidin-conjugated agarose beads. Following centrifugation, the relative level of biotinylated protein and E2 subunit present in the supernatant was evaluated by Western blot analysis utilizing HRP-conjugated avidin and anti-KGDH E2 subunit antibodies, respectively. B. Proteins were derivatized with the reducible probe biotin-HPDP and biotinylated protein bound to avidin-conjugated agarose beads as described in part A. Non-biotinylated protein (supernatant) and biotinylated protein liberated from the avidin-conjugated agarose beads upon treatment with DTT were evaluated by Western blot analysis using polyclonal anti-KGDH E2 subunit antibodies. The blots shown are representative of three separate rats for each experimental protocol.

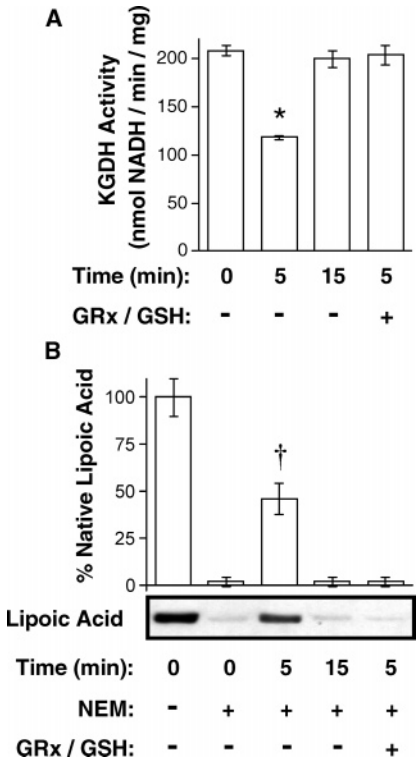



FIGURE 3: Immunochemical detection of $H_2O_2$-induced glutathionylation of KGDH lipoic acids. Isolated cardiac mitochondria (0.5 mg/mL) were treated with $H_2O_2$ (25 $\mu$M) in the presence of 5.0 mM α-ketoglutarate as respiratory substrate. At 0, 5, and 15 min, mitochondria were solubilized with 0.05% Triton X-100. A. KGDH activity was measured. Where indicated, samples were treated with glutaredoxin (1.0 U/mL) and GSH (0.5 mM) prior to enzyme analysis. Data represents the mean and standard deviation of results obtained using four separate mitochondrial preparations. *P* values (determined from a two tailed *t* test where value denoted by symbol is compared with each of the other values): * ≤ 0.0001. B. Mitochondrial samples were treated with NEM (10 mM) for 10 min to derivatize reduced sulfhydryl groups on protein. Unreacted NEM was scavenged with NAC (20 mM). Where indicated, samples were treated with glutaredoxin (1.0 U/mL) and GSH (0.5 mM) prior to NEM derivitization. Samples (5.0 $\mu$g protein/lane) were then evaluated by gel electrophoresis (reducing conditions, 100 mM DTT) and Western blot analysis using polyclonal anti-lipoic acid as primary antibody. The level of anti-lipoic acid binding was quantified by densitometric analysis. Data represents the mean and standard deviation of results obtained using four separate mitochondrial preparations. *P* values (determined from a two tailed *t* test where value denoted by symbol is compared with each of the other values): † ≤ 0.001.

protein sulfhydryls exposed upon treatment with glutaredoxin and GSH. In this manner, biotinylated protein can be eluted from agarose-immobilized streptavidin upon incubation with the reducing agent DTT. As shown in Figure 2B, E2 was specifically biotinylated (glutathionylated) under conditions where KGDH was maximally inhibited (exposure to $H_2O_2$ for 5.0 min) and this process was completely reversible. The E2 subunit of KGDH is therefore glutathionylated and reversibly inhibited when mitochondria are challenged with $H_2O_2$.

The E2 subunit of KGDH contains a lipoic acid residue covalently linked to the $\epsilon$-amino group of a specific lysine residue. Enzyme catalysis requires cycling of the two sulfur atoms on lipoic acid between reduced and oxidized states (*25*). Lipoic acid therefore represents a likely site of glutathionylation. To test this possibility, antibodies specific to lipoic acid were utilized. These antibodies do not recognize lipoic acid when sulfhydryl groups are derivatized with NEM prior to Western blot analysis (Figure 3). If the site of glutathionylation is lipoic acid, NEM modification would be reduced (Table 1) preserving recognition of lipoic acid upon Western blot analysis under reducing conditions that remove glutathione and regenerate native lipoic acid. Mitochondria were therefore incubated with $H_2O_2$ for specified periods of time followed by treatment with NEM. Subsequent Western blot analysis revealed lipoic acid antibody binding exclusively in samples in which KGDH had been inhibited by $H_2O_2$ (5.0 min) with loss of antibody binding evident upon enzyme reactivation. The level of binding relative to non-NEM treated samples (~50%) was consistent with the degree of enzyme inhibition (Figure 3). Treatment of $H_2O_2$-exposed mitochondria with glutaredoxin and GSH to remove glutathione and regenerate reduced sulhydryl groups prior to NEM derivatization resulted in complete loss in antibody recognition (Figure 3). Thus, upon incubation of mitochondria with $H_2O_2$, lipoic acid on the E2 subunit of KGDH undergoes reversible glutathionylation resulting in transient loss in enzyme activity. Reduced lipoic acid contains two sulfhydryl groups. While glutathionylation of either sulhydryl may sterically hinder modification of the other, the precise position and stoichiometry of glutathionylation remains to be determined.

Apart from enzyme inhibition and modulation of mitochondrial respiratory activity, glutathionylation of KGDH

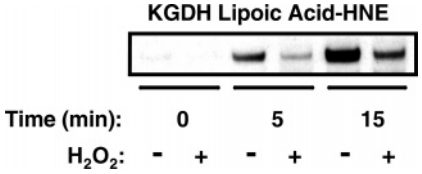
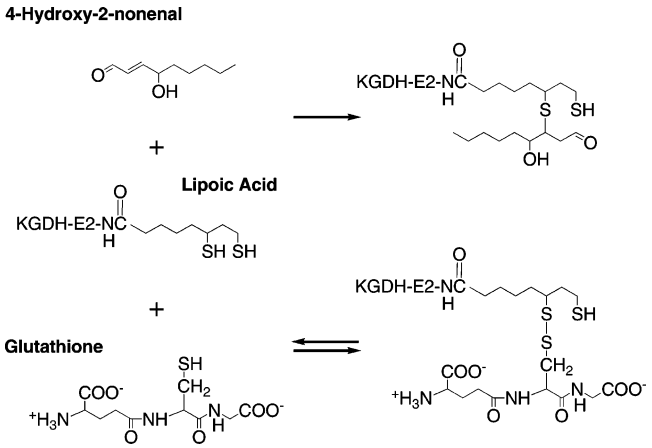



FIGURE 4: Effect of glutathionylation on the susceptibility of lipoic acid on KGDH to modification by HNE. Isolated cardiac mitochondria respiring on 5.0 mM α-ketoglutarate were incubated in the absence or presence of $H_2O_2$ (100 $\mu$M). After 2.0 min, HNE (100 $\mu$M) was added and incubations were allowed to proceed for 0, 5, and 15 min. Unreacted HNE was scavenged upon addition of NAC (10 mM), and samples were reduced with $NaBH_4$. Western blot analysis (10 $\mu$g of protein/lane) utilizing anti-lipoic acid-HNE was then perfomed.

Scheme 1: Glutathionylation Protects Lipoic Acid from Modification by 4-Hydroxy-2-nonenal




may serve to protect lipoic acid from irreversible oxidative damage. 4-Hydroxy-2-nonenal (HNE) is a highly reactive and cytotoxic product formed when polyunsaturated fatty acids are oxidized (*26*, *27*). We have previously demonstrated that treatment of cardiac mitochondria with HNE results in loss in respiratory activity that is directly attributable to specific inactivation of KGDH (*16*, *17*). Inactivation was the result of the unique sensitivity of lipoic acid residues on KGDH to HNE modification (*16*, *17*). We therefore sought to determine whether glutathionylation of KGDH protects from HNE modification. Antibodies have been prepared that recognize the lipoic acid−HNE adduct and exhibit no cross reactivity with adducts formed between HNE and amino acids (cysteine, histidine, or lysine). As shown in Figure 4, treatment of mitochondria with HNE resulted in a time dependent increase in HNE modified lipoic acid. Treatment of mitochondria with $H_2O_2$ prior to the addition of HNE resulted in protection of the lipoic acid residues from HNE modification (Figure 4). Thus, glutathionylation of lipoic acid on KGDH may preserve the capacity of the enzyme to withstand greater and/or prolonged oxidative insult(s) (Scheme).

## DISCUSSION

Insight on redox regulation of protein function will be gained through the thorough identification of (1) proteins that are reversibly altered in response to changes in redox status; (2) mechanisms by which these events are controlled and integrated with other regulatory processes; and (3) metabolic consequences of redox-dependent modulation in function. We previously identified KGDH as an enzyme that undergoes reversible glutathionylation and inhibition in response to treatment of mitochondria with $H_2O_2$ (*13*, *14*). KGDH limits the rate of certain functional parameters of the mitochondria and is regulated by a variety of metabolites (*15*−*19*, *25*). As such, KGDH is an important enzyme with which to delineate mechanisms and assess ramifications of redox regulation. In the current study, we have identified the enzyme's cofactor lipoic acid, covalently linked to the E2 subunit, as the site of glutathionylation. The relative level of E2 subunits modified parallels the degree of enzyme inhibition indicating that glutathionylation is solely responsible for inhibition of activity. In addition, glutathionylation and enzyme inhibition are both fully reversible. The E2 subunit of KGDH is clearly not the only protein that undergoes reversible glutathionylation when cardiac mitochondria are treated with $H_2O_2$ (Figure 1). Future studies that identify other glutathionylated proteins will expand the current understanding of redox regulation of mitochondrial function.

While mechanism(s) of protein glutathionylation remain to be elucidated, glutathionylation may involve bimolecular collision between GSSG and protein sulfhydryl groups and/or pro-oxidant priming of the sulfhydryl on GSH or the target protein to produce potent nucleophiles. Specificity may then be governed by the presence of low p$K_a$ cysteines (*7*, *28*, *29*). In addition, glutathionylation may occur via enzymatic pathway(s) (*3*, *4*, *6*, *14*). While deglutathionylation of KGDH is readily accomplished upon addition of glutaredoxin, glutathionylation and inhibition of KGDH have proven difficult to reconstitute. Mitochondria must remain intact and be actively respiring for $H_2O_2$ to induce inhibition of KGDH (*14*). Treatment of disrupted mitochondria or purified KGDH with various combinations of $H_2O_2$, GSH, GSSG, and enzyme substrates and cofactors has failed to result in reversible glutathionylation and inhibition of KGDH (*14*). Finally, pyruvate dehydrogenase, which resembles KGDH in cofactor requirements, subunit composition, and enzymatic mechanism, does not undergo reversible glutathionylation when mitochondria are treated with $H_2O_2$ under conditions tested to date. These observations raise the possibility that not simply deglutathionylation, but also glutathionylation involves an enzymatic process much like phosphatase and kinase catalyzed protein dephosphorylation and phosphorylation (*4*, *6*, *14*). Advantages would include the high degree of specificity afforded and the potential for exquisite regulation and coordinated interactions with other modulatory systems and metabolites. The current study identifies the subunit and site of KGDH glutathionylation, information necessary to identify and characterize processes that are responsible.

The reaction pathway catalyzed by KGDH, decarboxylation of α-ketoglutarate and reduction of $NAD^+$ to NADH, takes advantage of the unique properties of lipoic acid (*25*). These same inherent properties, however, prime lipoic acid for nucleophilic addition to lipid peroxidation products such as HNE, thereby conferring susceptibility to irreversible forms of modification under conditions of oxidative stress (*16*, *17*). We have shown that glutathionylation protects the highly reactive and essential lipoic acid residues from more severe forms of oxidative modification (Scheme 1). An additional facet is the protection afforded vicinal sulfhydryl

groups on the E3 subunit which cycle between reduced and oxidized states during enzyme catalysis. Glutathionylation of lipoic acid would prevent reduction of the disulfide on E3 and, thus, oxidative damage of reduced sulfhydryl groups. Glutathionylation of KGDH can therefore be viewed as an appropriate antioxidant response providing time for resolution of oxidative stress and subsequent recovery of enzyme activity. Nevertheless, if pro-oxidant production persists or is of sufficient magnitude, the biochemical nature of reversible inhibition (cycling between inhibited and active/susceptible states) may retard but not prevent the eventual progression to irreversible inactivation. Redox-dependent modulation of KGDH would, however, be expected to limit the production of NADH and entry of electrons into a compromised electron transport chain, thereby limiting the duration and extent of oxidative stress.

It is perhaps myopic to consider redox-dependent inhibition of KGDH solely as a response that serves to minimize oxidative damage. Under the conditions of our experiments, KGDH activity was maximally reduced to 50% of control values as previously described (*13*, *14*). The inability to provoke complete loss of activity may be explained by rapid cycling between glutathionylation states of the enzyme and/or the existence of KGDH in distinct microenvironments. Alternatively, KGDH is a key regulatory enzyme with changes in activity affecting NADH production and, in turn, mitochondrial membrane potential, oxidative phosphorylation, and ion transport. Thus, partial inhibition of KGDH may reflect the coordinated regulation of mitochondrial function through redox-dependent and -independent mechanisms.

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