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Reversible inactivation of dihydrolipoamide dehydrogenase by mitochondrial hydrogen peroxide

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Abstract

Under oxidative stress conditions, mitochondria are the major site for cellular production of reactive oxygen species (ROS) such as superoxide anion and H_2O_2 that can attack numerous mitochondrial proteins including dihydrolipoamide dehydrogenase (DLDH). While DLDH is known to be vulnerable to oxidative inactivation, the mechanisms have not been clearly elucidated. The present study was therefore designed to investigate the mechanisms of DLDH oxidative inactivation by mitochondrial reactive oxygen species (ROS). Mitochondria, isolated from rat brain, were incubated with mitochondrial respiratory substrates such as pyruvate/malate or succinate in the presence of electron transport chain inhibitors such as rotenone or antimycin A. This is followed by enzyme activity assay and gel-based proteomic analysis. The present study also examined whether ROS-induced DLDH oxidative inactivation could be reversed by reducing reagents such as DTT, cysteine, and glutathione. Results show that DLDH could only be inactivated by complex III- but not complex I-derived ROS; and the accompanying loss of activity due to the inactivation could be restored by cysteine and glutathione, indicating that DLDH oxidative inactivation by complex III-derived ROS was a reversible process. Further studies using catalase indicate that it was H_2O_2 instead of superoxide anion that was responsible for DLDH inactivation. Moreover, using sulfenic acid-specific labeling techniques in conjunction with two-dimensional Western blot analysis, we show that protein sulfenic acid formation (also known as sulfenation) was associated with the loss of DLDH enzymatic activity observed under our experimental conditions. Additionally, such oxidative modification was shown to be associated with preventing DLDH from further inactivation by the thiol-reactive reagent N-ethylmaleimide. Taken together, the present study provides insights into the mechanisms of DLDH oxidative inactivation by mitochondrial H_2O_2 .

Keywords

brain; dihydrolipoamide dehydrogenase; H_2O_2 ; mitochondria; reactive oxygen species; reversible inactivation; sulfenic acid; sulfenation

Introduction

Dihydrolipoamide dehydrogenase (DLDH) is the third catalytic enzyme of mitochondrial α -keto acid dehydrogenase complexes including pyruvate dehydrogenase complex, α -

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

ketoglutarate dehydrogenase complex, and branched chain amino acid dehydrogenase complex [1]. It is also a component in the glycine cleavage system [2]. In vivo, DLDH catalyzes the reoxidation of dihydrolipoamide using NAD^+ as the electron acceptor [3]. In vitro, however, the enzyme can also catalyze the reduction of lipoamide back to dihydrolipoamide using NADH as the electron donor [3]. DLDH also possesses diaphorase activity that can transport electrons from NADH to a variety of artificial electron acceptors such as nitro blue tetrazolium (NBT) [4] and dichlorophenolindophenol (DCPIP) [3].

DLDH is a redox-sensitive, multifunctional oxidoreductase and is very susceptible to oxidative modification. It has been reported that DLDH can be attacked by lipid peroxidation byproducts [5], can be nitrated by reactive nitrogen species [6,7], and can also be carbonylated by reactive oxygen species (ROS) [8]. DLDH has two redox-reactive cysteine residues at its active center that are indispensable for its catalytic function [2,9]. However, as redox-reactive cysteine residues are susceptible to oxidative modifications [10–12], the two cysteine residues could also render DLDH vulnerable to oxidative inactivation [4,13]. Indeed, it has been reported that DLDH can undergo poly(ADP-ribosyl)ation and s-nitrosylation [14–19] by reactive nitrogen species under a variety of experimental conditions. Additionally, depending on experimental systems, DLDH not only can augment oxidative stress [20–27], but can also attenuate oxidative stress [28,29]. Interestingly, DLDH may also play a role in protective response as its expression has been shown to be significantly upregulated, respectively, in estrogen signaling [30] and in human heart failure [31].

Mitochondria are the major source of endogenous ROS [32] and both complexes I and III in the electron transport chain have been recognized as the enzyme systems that generate ROS [33]. While DLDH from a variety of organisms can be oxidatively modified with a loss in enzyme activity by exogenous oxidants [34–37], the mechanisms of its inactivation by endogenous, mitochondria-generated ROS remain unknown. Moreover, in proteomic studies whereby DLDH was identified to be oxidatively modified [5 – 8,15], no effort was ever made to examine the effects of such modifications on enzyme activity. The purpose of this study was, therefore, to elucidate the mechanisms of DLDH oxidative inactivation by mitochondrial ROS. Specifically, using isolated mitochondria as a stand-alone system, we wanted to address whether DLDH is sensitive to oxidative inactivation by complex I- or complex III-derived ROS, whether the inactivation is reversible, and what would be the actual reactive species and the nature or the adduct of the oxidative modifications.

Materials and methods

Animals and chemicals

Adult male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) were used throughout the studies. Bis-Tris was purchased from Calbiochem (La Jolla, CA, USA). Tricine and ϵ -amino-*N*-caproic acid were purchased from MP Biochemicals. Acrylamide/bisacrylamide, ammonium persulfate, and CBB R-250 were purchased from Bio-Rad laboratories (Richmond, CA, USA). BSA, NADH, EDTA, lipoamide, *N*-ethylmaleimide (NEM) and biotin-NEM, cysteine, glutathione (GSH), sodium arsenite, and NBT chloride tablets were purchased from Sigma (St. Louis, MO, USA). Serva Blue G was from Serva (Heidelberg, Germany). Bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL, USA). Rabbit anti-DLDH polyclonal antibodies (IgG) were from US Biological (Swampscott, MA, USA) and goat anti-rabbit IgG conjugated with horseradish peroxidase were from Invitrogen (San Diego, CA, USA). Hybond-C membrane and an ECL immunochemical detection kit were obtained from GE Healthcare (Piscataway, NJ, USA). Dihydrolipoamide was prepared using sodium borohydride reduction of lipoamide as

previously described [3,38]. DCP-Bio-1, a specific sulfenic acid probe [39], was purchased from KetaFAST (Boston, MA).

Preparation of brain mitochondria

Rat brain mitochondria were isolated as previously described [40] with slight modifications [4,13]. Briefly, brains were removed rapidly and homogenized in 15 ml of ice-cold, mitochondrial isolation buffer containing 0.32 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.1. The homogenate was centrifuged at 1,330 g for 10 min and the supernatant was saved. The pellet was resuspended in 0.5 (7.5 ml) volume of the original isolation buffer and centrifuged again under the same conditions. The two supernatants were combined and centrifuged further at 21,200 g for 10 min. The resulting pellet was resuspended in 12% Percoll solution that was prepared in mitochondrial isolation buffer and centrifuged at 6,900 g for 10 min. The resulting supernatant was then carefully removed by vacuum. The obtained soft pellet was resuspended in 10 ml of the mitochondrial isolation buffer and centrifuged again at 6,900 g for 10 min. All isolated mitochondria were used fresh and protein concentrations were determined by bicinchoninic acid assay [41].

In vitro mitochondrial oxidative stress

Mitochondrial oxidative stress *in vitro* was induced by supplementing mitochondria with respiratory substrates such as pyruvate/malate or succinate in the presence of electron transport chain inhibitors such as rotenone or antimycin A, a condition that is known to enhance mitochondrial ROS generation [13,42,43]. Mitochondrial incubation was carried out as previously described [44]. Briefly, mitochondria (0.25 mg/ml) were incubated at 25°C for 60 min in incubation buffer (110 mM mannitol, 10 mM KH₂PO₄, 60 mM Tris, 60 mM KCl, and 0.5 mM EGTA, pH 7.4) in the presence of 50 μM rotenone or 50 μM antimycin A. The mixture was then supplemented with either complex I substrates pyruvate/malate (5 mM each) or complex II substrate succinate (10 mM). Control mitochondria were incubated under the same conditions in the absence of any substrates and inhibitors. At the end of the incubation, mitochondria were pelleted by centrifugation at 8,000 g for 10 min followed by enzyme assays or sulfenic acid labeling. For further incubation of the mitochondrial samples with reducing reagents such as DTT, cysteine, and GSH, 10 mM of each of the chemicals was added to the mixture and the sample was incubated at room temperature for an additional 30 min followed by measurement of enzyme activities. For evaluation of the effect of catalase on DLDH oxidative modification induced by antimycin A/succinate, broken mitochondria, prepared by resuspending in 50 mM potassium phosphate buffer (pH 7.4) followed by sonication, were used so that catalase can readily get access to any formed hydrogen peroxide.

Measurement of total ROS and superoxide anion

Levels of total mitochondrial ROS following incubation with substrates and electron transport chain inhibitors were measured by the fluorescence probe DCFH as previously described with slight modifications [45]. Following *in vitro* oxidative stress, 1 μM DCFH (in dimethyl sulfoxide) was incubated with 1 ml of 50 μg mitochondrial proteins for 1 hr at room temperature. Fluorescence intensity was read by a fluorometer equipped with a 96-well plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Measurement of superoxide anion by MitoSOX (from Invitrogen) was performed as previously described [46]. Additionally, superoxide generation by sub-mitochondrial particles was also measured by the method that involves SOD-inhibitable reduction of acetylated cytochrome c [47].

Determination of enzyme activities

Dehydrogenase activity was measured by DLDH catalyzed, NAD^+ -dependent oxidation of dihydrolipoamide [3,38]. The final volume of reaction was 1 ml, and the mixture contained 100 mM potassium phosphate, pH 8.0, 1.0 mM EDTA, 0.6 mg/ml BSA, 3.0 mM NAD^+ , 5–10 $\mu\text{g/ml}$ mitochondrial extract and 3.0 mM dihydrolipoamide. A solution containing all the assay components except dihydrolipoamide was used as the blank. The reaction was initiated by the addition of dihydrolipoamide and the change in absorbance at 340 nm was followed at room temperature. DLDH diaphorase activity was performed using blue native polyacrylamide gel electrophoresis (BN-PAGE) as previously described [4].

Labeling of protein sulfenic acids with biotin-maleimide

Labeling and detection of protein sulfenic acids was performed as previously described [48]. Mitochondrial pellet, immediately following *in vitro* incubation, was solubilized in a thiol-group blocking buffer containing 100 mM sodium acetate (pH 7.0), 20 mM NaCl, 1% SDS and 100 mM N-ethylmaleimide (NEM). The protein mixture was incubated on a rotator at room temperature for 2 hrs followed by clarification of the mixture by centrifugation at 13,000 g for 10 min. Excess NEM in the supernatant was removed by gel filtration using PD-10 columns. This was followed by addition of 0.1 mM biotin-maleimide and 20 mM sodium arsenite (both final concentrations) to the eluate. The sample was further incubated on a rotator at room temperature for 30 min. Proteins were then precipitated by 10% TCA (final concentration) on ice for 10 min followed by centrifugation on a bench top centrifuge at 3,000 rpm for 5 min. The pellet was washed three times with ethyl acetate: ethanol (1:1, v/v). Protein pellet after the third wash was used for polyacrylamide gel electrophoresis as described below.

Polyacrylamide gel electrophoresis and Western blot analysis

One dimensional Laemmli SDS-PAGE (7.5% resolving gel) was performed except that the running buffer used was that of Tricine SDS-PAGE (100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3) [49]. Two dimensional-PAGE was performed as previously described [19,50]. Proteins on gels were stained with Coomassie colloid blue [51], and Western blot analysis was performed according to standard procedures.

Mass spectrometry analysis of DLDH cysteine modifications

For MS analysis of cysteine oxidative modifications in DLDH, gel bands resolved by BN-PAGE were excised and destained with 50 mM ammonium bicarbonate prepared in 50% methanol. This was followed by rehydration and trypsin digestion according to standard procedures. Peptides were analyzed using nano-LC/MS/MS system comprising a nano-LC pump and a LTQ-FT mass spectrometer (Thermo Electron Corporation, San Jose, CA) equipped with a nanospray ion source. Approximately 5 to 20 fmoles of tryptic digest samples were dissolved in 5% acetonitrile with 0.1% formic acid and injected onto a C18 nanobore LC column for nano-LC/MS/MS and identification of peptides. A linear gradient LC profile was used to separate and elute peptides with a constant total flow rate of 350 nL/minute. The gradient consisted of 5 to 70% solvent B in 78 minutes (solvent B: 80% acetonitrile with 0.1% formic acid; solvent A: 5% acetonitrile with 0.1% formic acid). Mascot database searches were performed using Bioworks Browser 3.2 software (Thermo Electron Corporation, San Jose, CA). Identified peptides were generally accepted only when the MASCOT ion score value exceeded 20.

Gel image documentation and data analysis

All images were scanned by an EPSON PERFECTION 1670 scanner. Densitometric analysis of gel bands was performed using Image J software. For enzyme activities, data

were presented as mean $\pm$ SEM of triplicate assays. $P < 0.05$ was considered significant using Welch's t-test.

Results

DLDH inactivation by complex III-derived ROS

To investigate the mechanisms of DLDH inactivation by mitochondrial ROS, isolated mitochondria were incubated with respiratory substrates in the presence of inhibitors of electron transport chain. This approach is an *in vitro* incubation system that is known to elevate mitochondrial ROS production [13,42,43]. In the present study, pyruvate/malate were used as complex I substrates and succinate was used as complex II substrate. The inhibitors used were rotenone for complex I and antimycin A for complex III, respectively. Complex III inhibition by antimycin A would cause ROS production by both complex III and complex I as this inhibition would cause accumulation and leakage of electrons at both of the two complexes [33,52]. In contrast, complex I inhibition by rotenone would only induce ROS production by complex I as electrons only accumulate and leak at complex I [33,52]. Following incubation, mitochondrial extracts were prepared and DLDH activities were measured. Results show that, regardless of the substrates that were used, DLDH only exhibited inactivation of its dehydrogenase activity when antimycin A was present (Figure 1A) and antimycin A itself (50 μ M), in the absence of a respiratory substrate, did not impair DLDH activity (Figure 1B). Additionally, DLDH diaphorase activity analyzed by BN-PAGE shows a similar inactivation pattern to that of dehydrogenase activity (Figure 2). These results indicate that DLDH could only be inactivated by complex III-derived ROS under our experimental conditions.

The above observation that DLDH could only be inactivated by complex III-derived ROS suggests that complex I either does not produce ROS or produces ROS that can not get access to DLDH. To determine which of the two situations actually occurred in our experiments, we measured mitochondrial ROS by DCFH assay following the same incubation conditions as described in Figures 1 and 2. Results in Figure 3A show that both complexes I and III produced a significant amount of ROS. These results indicate that complex I indeed produced ROS in the presence of rotenone or antimycin A, but these ROS could not inactivate DLDH. Additionally, when the superoxide-specific probe MitoSox [46] was used to measure the rate of superoxide production, it was found that the rate of superoxide production was linearly correlated with antimycin A-concentrations (Figure 3B) whereby succinate concentration (10 mM) remained constant. Moreover, this linear relationship was further confirmed when mitochondrial superoxide production was measured by the method involving SOD-inhibitable reduction of acetylated cytochrome c using submitochondrial particles as the source of electron transport chain [47] (Figure 3C), which further demonstrates an authentic involvement of superoxide anion in our experimental system.

DLDH is inactivated by H_2O_2

It is well known that the initial reactive oxygen species generated by complex III, when stimulated by AA/succinate, is superoxide anion [42]. In the presence of mitochondrial superoxide dismutase whereby whole mitochondrial extract is used, the released superoxide can be quickly dismutated to yield H_2O_2 [52]. On the other hand, for a cysteine residue to undergo oxidative modification, it has to exist in the thiolate anion form at physiological pH [53]. Therefore it is unlikely that superoxide anion that owns a negative charge can directly attack a thiolate anion that is also negatively charged. We reasoned that it is H_2O_2 that inactivates DLDH under our experimental conditions. To test this possibility, we incubated broken mitochondria with antimycin A and succinate in the presence of catalase (1 mg/ml),

which was followed by DLDH enzyme activity assay. Result in Figure 4A shows that catalase completely abolished DLDH inactivation, demonstrating that DLDH was indeed inactivated by H_2O_2 under our experimental conditions.

DLDH inactivation by H_2O_2 can be reversed by cysteine and glutathione

To determine whether DLDH inactivation by complex III-generated H_2O_2 was reversible or not, we further added reducing reagents to the mitochondria incubation mixture. We chose DTT, cysteine, and glutathione as our reducing reagents as we suspected that the inactivation could be due to DLDH cysteine modifications. Results in Figure 4B show that DLDH inactivation could be completely reversed by cysteine and GSH, but not by DTT. Similarly, loss of DLDH diaphorase activity could also be restored by cysteine and GSH (Figure 5). These results suggest that DLDH inactivation by complex III H_2O_2 is caused by its cysteine modifications and is a reversible process.

DLDH inactivation by H_2O_2 involves sulfenic acid formation

Based on facts that glutathione reductase is structurally similar to DLDH [1] and glutathione reductase undergoes sulfenation under oxidative stress conditions [11], we suspected that our observed reversible DLDH inactivation by H_2O_2 may also be caused by protein sulfenic acid formation or sulfenation as this modification is a reversible process that can regulate protein function [54,55]. To determine whether this is the case, we first took a biotin switch approach [48] whereby protein sulfenic acids were reduced by arsenite back to free cysteine residues and the newly generated cysteine thiol groups were then labeled by biotin-maleimide (NEM). This was followed by 2D analysis of biotinylated proteins and DLDH localization by anti-DLDH Western blot. Results in Figure 6 show that DLDH was indeed sulfenated in the presence of succinate and antimycin A (panel 3). Additionally, we demonstrated that the arsenite-reducing method is indeed specific toward protein sulfenic acids as arsenite treatment performed after pre-incubation of samples with dimedone, a sulfenic acid specific reagent [56], largely abolished the immunoblot signals (panel 4, the middle lane).

Loss of DLDH activity correlates with increase in the level of DLDH sulfenation

If sulfenation is involved in the loss of DLDH activity, a correlation between loss of DLDH activity and increase in DLDH sulfenation would be expected. To determine whether this is the case, we then incubated mitochondrial samples with a sulfenic acid-specific probe DCP-Bio1 [39] after oxidative stress triggered by AA/succinate. This was followed by measurement of DLDH activity and separation of DLDH from other mitochondrial proteins by BN-PAGE [4]. The corresponding DLDH band on blue native gel was then excised and further analyzed by Western blot probed with streptavidin-HRP followed by densitometric quantitation of the signal intensities. Figure 7A shows a significant inactivation of DLDH by the system of AA/succinate; Figure 7B shows comparison of Western blot analysis of DLDH sulfenation between control and AA/succinate-treated samples; Figure 7C shows quantitatively that there was a significant increase in the level of sulfenation after treatment with AA/succinate. Taken together, these results indicate that loss of DLDH activity is inversely related with increase in the level of DLDH sulfenation.

Mass spectrometry analysis of DLDH cysteine residues that are oxidized under oxidative stress conditions

To determine which cysteine residues in DLDH underwent oxidative modifications that might be involved in the loss of enzyme activity, we performed mass spectrometry (MS) analysis. Following BN-PAGE resolution of both control and oxidized samples, DLDH-containing gel bands were excised and subjected to MS analysis. Results show that no

sulfenic acid or sulfinic acid formation could be detected. In contrast, sulfonic acid formation on cys-449 was detected for both control and oxidized samples; while that on cys-277 could only be detected in the oxidized samples. These results indicate that cys-449 likely underwent auto-oxidation during sample preparations and cys-277 was over-oxidized only after *in vitro* oxidative stress. Modifications of these two cysteine residues are unlikely to impair DLDH function as there have been no reports that cys-227 and cys-449 are redox-sensitive. It should be noted that the status of the two redox-sensitive cysteine residues (cys-45 and cys-50) at the enzyme's active center could not be analyzed as the peptides containing the two cysteine residues failed to be recovered.

DLDH oxidative inactivation by H₂O₂ is resistant to further inactivation by N-ethylmaleimide (NEM)

We have previously shown that DLDH can lose its activity upon treatment with thiol-reactive reagent such as NEM [4]. If DLDH oxidative inactivation in our preparation indeed occurred due to sulfenic acid formation on one of the two catalytic cysteine residues (Cys-45 or Cys-50) located at the active center, then this modification should prevent DLDH against further inactivation by NEM that is known to react primarily with either of the two cysteine residues [57–59]. To test this possibility, we treated mitochondria with varying concentrations of NEM following incubation with antimycin A/succinate. Result in Figure 8A shows that DLDH oxidative inactivation by mitochondrial H₂O₂ indeed protected DLDH from further inactivation by NEM. Interestingly, NEM could not further inactivate the enzyme even at a 2 mM concentration. The reason for this remains unknown, but it is not without precedent as glutathionylated α -ketoglutarate dehydrogenase is also resistance to further inactivation by NEM [60]. In contrast, such a resistance could not be observed when similar mitochondrial preparations were further treated by guanidine chloride that can induce protein denaturation [61] and is known to bind to multiple sites on DLDH [62] (Figure 8B). These results imply that DLDH sulfenation in our system indeed occurred on one of the two catalytic, redox-reactive cysteine residues.

Discussion

The major findings of the present study are that, under our experimental conditions using isolated mitochondria as a stand-alone system, DLDH could only be inactivated by H₂O₂ derived from complex III-, but not from complex I; and this inactivation could only be reversed by cysteine and GSH, but not by DTT. The present study has also demonstrated that DLDH inactivation by H₂O₂ likely involved protein sulfenic acid formation that could prevent DLDH from further alkylation by NEM. Additionally, as sulfenic acid formation is a reversible process [54], our results could also suggest that sulfenic acid formation may serve as a switch for DLDH activity under oxidative stress conditions.

It should be pointed out that in our *in vitro* oxidative stress system, both complexes I and III were capable of generating superoxide anion/H₂O₂ in the presence of anti-mycin A, regardless of the substrates that were supplemented (Figure 3). However, it was found that DLDH could only be inactivated by complex III-derived H₂O₂. Such results suggest that DLDH is physically associated with complex III. Indeed, when BN-PAGE-resolved DLDH gel band was processed by LC-Nano MS/MS for protein identification, ubiquinol cytochrome c reductase subunit 2 (a component of complex III) was found in the DLDH gel band while no complex I components could be detected [63]. Therefore, DLDH is only accessible to, and may only be inactivated by, complex III-derived H₂O₂. Alternatively, as it is well known that H₂O₂ can easily diffuse from one place to another in mitochondria, our observation that DLDH could not be inactivated by complex I-generated H₂O₂ suggests that there may be an indirect mechanism involving H₂O₂ but not by H₂O₂ directly.

Interestingly, while our data clearly demonstrate the reversibility of DLDH inactivation by mitochondrial H_2O_2 , our data also show that, among the reducing reagents that were tested, only cysteine and GSH could restore the enzyme's activity following oxidative inactivation. In contrast, DTT, a very efficient thiol protector [64], could not do so under our experimental conditions. These data are in agreement with previous findings that both GSH [65] and cysteine [66] can readily enter mitochondria, while DTT can not [67]. Therefore, DTT can not get access to the sulfenated cysteine residue(s) when intact mitochondria are oxidatively stressed. It should be noted that when pure DLDH isolated from pig heart was inactivated by metal-catalyzed oxidation, the inactivation could indeed be reversed by DTT, at least partially [68], indicating that DTT is able to get access to the oxidatively modified amino acid residues when purified DLDH is oxidized.

Rat DLDH contains a total of 10 cysteine residues [69]; and cys-45 and cys-50 are the two redox-sensitive cysteines located at the enzyme's active center [13]. During catalysis, cys-45 binds substrate dihydrolipoamide while cys-50 interacts with FAD [2]. While we were able to determine two cysteine residues (cys-277 and cys-449) that were sulfonated, and one of them (cys-277) was sulfonated only after *in vitro* oxidative stress, we were unable to determine the status of oxidative modifications to cys-45 and cys-50 because the peptides containing the two cysteines failed to be recovered following the procedures of mass spectrometry analysis. Nevertheless, our results that DLDH oxidative inactivation prevented against further inactivation by the thiol-reactive reagent NEM would suggest that sulfenation occurred on one of the two catalytic cysteine residues (Figure 8A). Additionally, based on studies that cys-45 is more reactive than cys-50 toward thiol-reactive reagents [59], we reason that cys-45 would undergo sulfenation under our experimental conditions. This reasoning would also be in agreement with the findings that, in glutathione reductase that is structurally similar to DLDH [1], only the substrate binding cysteine residue can undergo oxidative modification when challenged by reactive nitrogen species [11]. Nevertheless, further studies will be needed to determine which of the two redox-sensitive cysteine residues at the active center is modified under oxidative stress conditions.

Additionally, if sulfenation is the main cause of DLDH inactivation, there should be a positive correlation between the level of DLDH sulfenation and the loss in DLDH activity. Indeed, after mitochondrial oxidative stress by AA/succinate, loss of DLDH activity was found to be inversely correlated with an increase in the level of DLDH sulfenation (Figure 7). Furthermore, we also attempted to test such a correlation using partially purified DLDH that was incubated with varying concentrations of H_2O_2 followed by labeling with DCP-Bio1. DLDH was partially purified from rat liver according to methods previously described [70–72]. Enzyme activity was also measured spectrophotometrically after DCP-Bio1 labeling. Results in Supplementary Figure 1A to be found online at <http://informahealthcare.com/doi/abs/10.3109/10715762.2012.752078> show that while DLDH activity decreased further with increasing concentrations of H_2O_2 , DLDH sulfenic acid content, reflected by Western blot assay, actually decreased in an H_2O_2 concentration-dependent manner, indicating that purified DLDH had already been sulfenated and further oxidation by H_2O_2 over-oxidized the formed sulfenic acids, resulting in less and less labeling by DCP-Bio1 (Supplementary Figure 1B). In addition, while the maximum sulfenic acid signal intensity in the Western blot was obtained at 10 μ M H_2O_2 , nearly no inhibition of enzyme activity could be observed at this concentration of H_2O_2 (Supplementary Figure 1). Therefore, with the use of purified DLDH, assessment of a positive relationship between DLDH sulfenation and loss of enzyme activity did not yield expected results, presumably because of autooxidation during purification and overoxidation of protein sulfenic acids by H_2O_2 . Nonetheless, our studies using partially purified DLDH further indicate a genuine involvement of transitional sulfenic acid formation in DLDH oxidative modifications.

While it has been established that protein sulfenic acid plays a central role in protein thiol oxidation and is mainly produced by reactions between redox-sensitive cysteine residues and hydrogen peroxide, alkyl hydroperoxides, as well as peroxynitrite [48,54,55,73,74]; it is also known that sulfenic acid is a key intermediate that can generate the most commonly reversible cysteine modification products including protein disulfides, mixed disulfides or S-glutathionylation [75]. Nevertheless, there is now accumulating evidence that stabilized sulfenic acids do exist and play a key redox regulatory role in many aspects of cell biology [53,76]. For example, protein S-sulfenation has been found to be a prerequisite for T cell activation, proliferation and function [77]. With respect to DLDH sulfenic acid formation in our system, the reason why DLDH sulfenic acids are stable enough to be detected remains unknown. Nonetheless, the stability of DLDH sulfenic acids could be controlled by a variety of factors such as solvent access, steric hindrance, the lack of proximal thiols, nearby available hydrogen-bonding interactions and/or the presence of positively charged side chains [74,78].

Finally, it should be alerted that the precise physiological or pathophysiological role of DLDH sulfenation is unknown at this time and could only be speculated. It is probable that this reversible modification, when occurring on one of the two cysteine residues at the enzyme's active center, may protect DLDH from an irreversible oxidation and a permanent loss of enzyme activity (as shown in Figure 8A) provided that sulfenic acid would not be over-oxidized to form sulfinic or sulfonic acids that are irreversible. Moreover, DLDH sulfenation not only could act as a redox sink that preserves cellular antioxidant capacity, but could also serve as an on/off redox and metabolic switch used by cells to prevent against the occurrence of a widespread, severe, and irreversible cellular oxidative damage. Therefore, at early stage of oxidative stress, DLDH sulfenation is likely a trade-off mechanism by which mitochondria adapt to oxidative stress. It is also plausible that, under oxidative stress conditions whereby level of mitochondrial oxidant formation is elevated, DLDH inactivation via sulfenation could be part of mitochondrial oxidant-scavenging system in cellular metabolism, including mitochondrial superoxide dismutase and peroxiredoxins [79]. Hence, it is conceivable that DLDH sulfenation under oxidative stress conditions may be an adaptive or defensive response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) DLDH dehydrogenase activity following in vitro mitochondrial oxidative stress induced by electron transport chain inhibitors rotenone or antimycin A in the presence of pyruvate/malate (complex I substrates) or succinate (complex II substrate). * <0.05 vs control (no substrates). (B) Antimycin A (50 μM), in the absence of succinate, did not impair DLDH activity. Control samples contained only mitochondria. Incubation conditions were the same as those described for A.

Figure 2.

DLDH diaphorase activity following in vitro mitochondrial oxidative stress induced by electron transport chain inhibitors rotenone or antimycin A in the presence of pyruvate/malate (complex I substrates) or succinate (complex II substrate). A shows diaphorase activity staining by BN-PAGE; B shows densitometric results derived from A. * <0.05 vs control (no substrates).

Figure 3.

(A) Levels of mitochondrial ROS determined by DCFH assay. Following incubation of mitochondria under the indicated conditions, 50 μ g mitochondrial proteins was incubated with 1 μ M DCFH (prepared in DMSO) at room temperature for 60 min. Fluorescence was measured by a fluorometer equipped with a 96-well plate reader (excitation/emission: 485 nm/535 nm). * $p < 0.05$ vs control. (B) Mitochondrial superoxide production measured by MitoSox (excitation/emission: 510 nm/580 nm) exhibits an antimycin A concentration-dependent manner. The concentration of succinate was fixed at 10 mM. (C) Mitochondrial superoxide production measured by SOD-inhibitable acetylated cytochrome c reduction also exhibits an antimycin A concentration-dependent manner; submitochondrial particles (the membrane fraction) were used in this experiment as previously described [47].

Figure 4.

(A) Catalase abolished DLDH inactivation when broken mitochondria were incubated with 50 μ M antimycin A and 10 mM succinate for 30 min at room temperature; catalase used was 1 mg/ml. (B) Loss of dehydrogenase activity by H_2O_2 could be restored by cysteine and GSH. Following incubation of mitochondria with succinate and antimycin A, 10 mM of each of the three reducing reagents was added and the sample was further incubated at room temperature with gentle agitation for 30 min. DLDH dehydrogenase activity was measured as described in the text. * $p < 0.05$ vs control; ** $p < 0.05$ vs succinate/AA.

Figure 5.

Loss of diaphorase activity by H_2O_2 could be restored by cysteine and GSH. Following incubation of mitochondria with succinate and antimycin A, 10 mM of each of the three reducing reagents was added and the sample was further incubated at room temperature with gentle agitation for 30 min. DLDH diaphorase activity was measured as BN-PAGE based activity staining shown in A. B shows densitometric values derivatized from A. * $p < 0.05$ vs control; ** $p < 0.05$ vs succinate/AA.

Figure 6.

Two-dimensional gel electrophoretic analysis of DLDH sulfenation. Sulfenated proteins were treated with arsenite and labeled with biotin-NEM followed by 2D analysis. (1): a representative 2D map visualized by Coomassie blue staining; (2): pattern of protein sulfenation under control conditions; (3): pattern of protein sulfenation after mitochondrial incubation with succinate/antimycin A; (4): pre-incubation of mitochondrial samples with dimedone (the middle lane) could largely eliminate the immunoblot signals, demonstrating that the arsenite reducing method used in this study is indeed specific toward protein sulfenic acids. Biotinylated protein markers from Bio-Rad were used as the ladder. In both panels 1 and 3, A indicates the spot of DLDH.

Figure 7.

Loss of DLDH activity is correlated with increase in DLDH sulfenation. (A) Inactivation of DLDH after incubation with AA (50 μ M) and succinate (10 mM); control samples contain neither AA nor succinate. (B) DLDH sulfenation detected by DCP-Bio1. After incubation under the same conditions as described in A, mitochondrial samples were treated with DCP-Bio1. DLDH was separated by BN-PAGE and the corresponding DLDH band was excised for further resolution by SDS-PAGE followed by Western blot probed with streptavidin-HRP. Each group contains three samples and 25 μ g mitochondrial proteins was loaded onto blue native gel for each sample. (C) Densitometric quantitation of sulfenation signal intensity shown in B. * $p < 0.05$ vs control for both A and C.

Figure 8.

DLDH oxidative inactivation by mitochondrial H_2O_2 prevented against further inactivation by NEM (A) but not guanidine chloride (B). Mitochondrial oxidative stress and DLDH inactivation was induced by incubation with antimycin A/succinate as described in Figures 1 and 2. This was followed by incubation with varying concentrations of NEM (A) or guanidine chloride (B) as indicated in the respective panel. DLDH enzyme activity was then measured spectrophotometrically in the presence of dihydrolipoamide and NAD^+ .