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Quantification and identification of mitochondrial proteins containing vicinal dithiols ☆

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ABSTRACT

Vicinal dithiols may play a role in mitochondrial antioxidant defences and in redox signalling. We quantified protein vicinal dithiols within mammalian mitochondria using the vicinal dithiol-specific reagent phenylarsine oxide (PAO). We found 5–15% of thiols exposed on mitochondrial proteins were vicinal dithiols and that these thiols were particularly sensitive to oxidation by hydrogen peroxide. To visualise these proteins we used PAO to block vicinal dithiols, followed by alkylation of other thiols with *N*-ethylmaleimide (NEM). The PAO was then removed with 2,3-dimercapto-1-propanesulfonic acid (DMPS) and the exposed vicinal dithiols were labelled with iodoacetamide-biotin. To identify these proteins, we developed a selective proteomic methodology, based on Redox difference in gel electrophoresis (Redox-DIGE). Vicinal dithiol proteins were selectively labelled with a red fluorescent thiol-reactive Cy5 maleimide and mixed with Cy3 maleimide labelled protein in which vicinal dithiols remained untagged. Individual proteins were resolved by 2D gel electrophoresis and fluorescent scanning revealed vicinal dithiol proteins by the increase in Cy5 red fluorescence. These proteins were identified by peptide mass fingerprinting and mass spectrometry. These findings are consistent with roles for mitochondrial vicinal dithiol proteins in antioxidant defence and redox signalling and these methodologies will enable these roles to be explored.

Introduction

Cysteine residues within proteins contain thiols that have many important functional roles, for example in enzyme active sites, in iron-sulfur centers and in zinc finger motifs [1–5]. In addition, a number of thiols are present on the surfaces of native proteins and are exposed to the aqueous phase [1,3–6]. These solvent-exposed protein thiols can interact with reactive oxygen species (ROS[1]), reactive nitrogen species (RNS) and alkylating agents [6–8]. Generally, interactions with ROS or RNS convert an exposed thiol to a transiently modified version such as a sulfenic acid, a thiyl radical or an *S*-nitrosothiol [9–12]. A sulfenic acid or a thiyl radical can be recycled back to a reduced state by reaction with glutathione (GSH) to form a mixed disulfide that can be reduced by glutaredoxin (Grx), or by thioredoxin (Trx) [9–12]. An *S*-nitrosthiol can be converted to a mixed disulfide or to a vicinal disulfide by reaction with a thiolate that displaces the nitroxyl anion [8]. If the transiently modified protein thiols are not recycled then they can become irreversibly oxidised to higher thiol oxidation states such as sulfinic or sulfonic acids [6,13]. Thus the rapid recycling of protein surface thiols protects proteins from oxidative damage and may also contribute to general antioxidant defences [6,14]. This ability of surface thiols to respond reversibly to the local redox environment also enables certain cysteine residues to act as nodes of redox signalling [1,8,13,15].

The majority of exposed protein thiols exist as isolated, single thiols. However, a number of protein thiols are sufficiently close to one another so that when oxidised they are able to form an intraprotein disulfide [15,16]. These groups, termed vicinal dithiols, can occur through proximity in the primary sequence, typically a $-CX_nC-$ motif where n is usually in the range 2–6 [16]. Alternatively, these cysteine residues can be separated in the primary sequence but close together in the tertiary structure. Vicinal dithiols are a particularly interesting subset of surface thiols because the effective high local thiol concentration (in the 100–500 mM range)

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[1] *Abbreviations used:* BSA, bovine serum albumin; BVA, biological variance analysis; DDM, *n*-dodecyl-β-D-maltopyranoside; DIGE, difference in gel electrophoresis; DMPS, 2,3-dimercapto-1-propanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfhide; GST, glutathione-*S*-transferases; PrSSG, protein mixed disulfhides with GSH; NEM, *N*-ethylmaleimide; PAO, phenylarsine oxide; Prx, peroxiredoxin; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDS, sodium dodecylsulfate; Trx, thioredoxin; TrxR, thioredoxin reductase.

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makes them more susceptible to oxidation to a disulfide than isolated thiols [1,4]. Thus a vicinal dithiol pair is more sensitive to oxidation by ROS/RNS during redox signalling, and possibly to oxidation by other dithiol proteins such as Trx or peroxiredoxin, perhaps facilitating protein redox signalling relays [17]. Furthermore, the proximity of a second thiol means that transient oxidation of one thiol in a vicinal dithiol to a thiyl radical, a sulfenic acid, or an *S*-nitrosothiol is likely to lead to the formation of a disulfide, which can be rapidly reduced back to a dithiol by the GSH and Trx systems. This contrasts with isolated protein thiols where formation of a thiyl radical or a sulfenic acid is often the first step to further irreversible oxidation to a sulfinic or sulfonic acid if the transiently oxidised thiol is not recycled by GSH. Thus, surface vicinal dithiols may be more effective at protecting proteins from oxidative damage than single thiols as well as potentially acting as sensitive nodes in redox signalling pathways (Fig. 1).

As mitochondria are a significant source of ROS, they are particularly susceptible to oxidative damage and are thought to be central to redox signalling [18–20]. Furthermore, within the mitochondrial matrix these exposed thiols are the most abundant free thiol present [6]. Consequently within mitochondria vicinal dithiols may make a significant contribution to the responses of mitochondria to both oxidative damage and redox signalling. However, little is known about the content and properties of vicinal dithiols within mitochondria. Here we set out to quantitate vicinal dithiols within mammalian mitochondria, to see if these vicinal dithiols respond to mild oxidative stress more readily that bulk protein thiols, and to develop proteomic methodologies to identify vicinal dithiol proteins.

Materials and methods

Preparation and incubation of mitochondria and membranes

Rat liver mitochondria were prepared by homogenisation followed by differential centrifugation in STE (250 mM sucrose, 5 mM Tris–HCl, 1 mM EGTA, pH 7.4) containing 0.1% (w/v) fat-free BSA at 4 °C [21]. Rat heart mitochondria were prepared by homogenisation using an Ultraturrax homogeniser and isolated by differential centrifugation in STE containing 0.1% (w/v) fat-free BSA. Mitochondrial protein content was determined by the biuret assay using BSA a standard [22]. Mitochondrial membranes were prepared by disruption of bovine heart mitochondria in a blender, followed by collection and washing by centrifugation [23]. These mitochondrial membrane preparations had negligible matrix contamination and were open fragments of mitochondrial membranes [24]. Prior to experiments mitochondrial membranes were preincubated at 20 mg protein/ml with 1 mM DTT in membrane buffer (20 mM Tris, 1 mM EDTA, pH 7.3) for 10 min at 37 °C. The membranes were then pelleted by centrifugation (10,000*g*) and washed twice in membrane buffer before use. Mitochondrial incubations were in KCl buffer (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.4) at 37 °C.

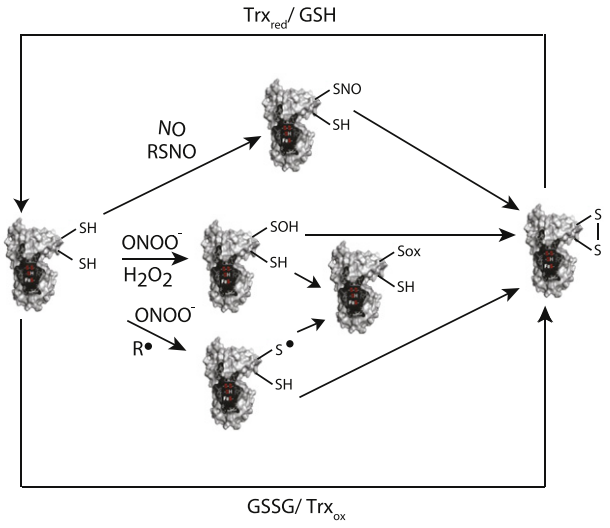

Fig. 1. Redox modification of protein vicinal dithiols. Once one thiol of a protein vicinal dithiol pair has been modified to form a thiyl radical, a sulfenic acid or an *S*-nitrosothiol it can be rapidly converted to an intraprotein disulfide due to the proximity of the second thiol of the pair. This makes the irreversible oxidation of the thiol to a sulfinic or sulfonic acid less likely than for a single thiol as well as making vicinal dithiols nodes for redox regulation. The intraprotein disulfide can then be recycled back to the reduced state by Trx/GSH.

Protein thiol assay

To measure exposed protein thiols in native proteins we dissolved mitochondria or mitochondrial membranes after incubation with or without the indicated concentrations of phenylarsine oxide (PAO) in 0.1% of the mild detergent *n*-dodecyl-β-D-maltopyranoside (DDM) to disrupt membranes without denaturing proteins [6]. This was followed by centrifugal gel filtration using a spin column (Micro Bio-Spin 6, Bio-Rad) to remove low molecular weight thiols such as GSH [6]. The thiol content was subsequently measured using the 5,5′-dithiobis(2-nitrobenzoic acid (DTNB) assay [25] and the protein content was measured using the bicinchoninic acid assay as described [6].

SDS–PAGE electrophoresis and immunoblotting

After incubation of mitochondria or membrane samples ±PAO, 1 mM *N*-ethylmaleimide (NEM) was added and the samples were pelleted by centrifugation. Pellets were then resuspended and reduced with 2 mM 2,3-dimercapto-1-propanesulfonic acid (DMPS) for 10 min at room temperature. DMPS was removed by centrifugal filtration, and the samples were resuspended in incubation buffer and labelled by reaction with 2 mM iodoacetyl-biotin (Pierce Biotechnology). Samples were separated by SDS–PAGE (5–12% acrylamide gradient) using a Bio-Rad Mini Protean System and transferred to 0.22 μm nitrocellulose membrane. The blot was incubated with streptavidin-horse radish peroxidase conjugate and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Redox-difference in gel electrophoresis (DIGE)

After incubation of mitochondrial samples with PAO, thiols were quenched by addition of 50 mM NEM and the mitochondria were pelleted by centrifugation. Pellets were then resuspended in assay medium containing 50 mM NEM and incubated for 5 min at 37 °C. At this point 1% SDS was added, and the mitochondrial lysate was incubated for a further 5 min. The NEM was removed using a Micro Bio-Spin 6 chromatography column (Bio-Rad), maintaining a 1% SDS concentration throughout. The samples were then incubated ±2 mM DMPS for 10 min at room temperature. DMPS was removed using two more Micro Bio-Spin 6 chromatography columns (Bio-Rad), and the samples were labelled with 40 μM CyDye™ DIGE Fluor Cy™3 saturation dye (GE Healthcare) or CyDye™ DIGE Fluor Cy™5 saturation dye (GE Healthcare). After 10 min at 37 °C, the reaction was quenched with 2.5 mM DTT, and the samples were snap frozen on dry ice prior to pooling and resolution by 2D electrophoresis. Resolution of mitochondrial proteins by 2D gel electrophoresis and scanning of gel fluorescence was as described previously [26,27].