Redox activation of mitochondrial intermembrane space Cu,Zn-superoxide dismutase

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The localization of Cu,Zn-superoxide dismutase in the mitochondrial intermembrane space suggests a functional relationship with superoxide anion (O$_2^-$•) released into this compartment. The present study was aimed at examining the functionality of Cu,Zn-superoxide dismutase and elucidating the molecular basis for its activation in the intermembrane space. Intact rat liver mitochondria neither scavenged nor dismutated externally generated O$_2^-$•, unless the mitochondrial outer membrane was disrupted selectively by digitonin. The activation of the intermembrane space Cu,Zn-superoxide dismutase following the disruption of mitochondrial outer membrane was largely inhibited by bacitracin, an inhibitor of protein disulphide-isomerase. Thiyl alkylating agents, such as N-methylmaleimide or iodoacetamide, decreased intermembrane space Cu,Zn-superoxide dismutase activation during, but not after, disruption of the outer membrane. This inhibitory effect was overcome by exposing mitochondria to low micromolar concentrations of H$_2$O$_2$ before disruption of the outer membrane in the presence of the alkylating agents. Moreover, H$_2$O$_2$ treatment alone enabled intact mitochondria to scavenge externally generated O$_2^-$•. These findings suggest that intermembrane space Cu,Zn-superoxide dismutase is inactive in intact mitochondria and that an oxidative modification of its critical thiol groups is necessary for its activation.

Key words: Cu,Zn-superoxide dismutase, hydrogen peroxide, mitochondrial intermembrane space, mitochondrion, superoxide anion.

INTRODUCTION

Mitochondria are major cellular sources of superoxide anion (O$_2$$^•$−) and hydrogen peroxide (H$_2$O$_2$) [1]. O$_2$$^•$−, generated by autoxidation of ubiquinone [2,3], is converted to H$_2$O$_2$ by the Mn-superoxide dismutase in the mitochondrial matrix. O$_2$$^•$− and H$_2$O$_2$ generated in mitochondria can also diffuse into the cytosol [4]. In addition to their potentially damaging effects on cellular constituents, O$_2$$^•$− and H$_2$O$_2$, at low levels, are essential participants in cell signalling events that regulate cell growth, differentiation and death [5]. Therefore pathways that maintain steady-state levels of intracellular O$_2$$^•$− and/or H$_2$O$_2$ have an important impact on the redox regulation of cell signalling and, as a corollary, cell function.

Early studies on the subcellular distribution of superoxide dismutase demonstrated that Mn-superoxide dismutase was localized in the mitochondrial matrix, whereas Cu,Zn-superoxide dismutase was only localized in cytosol [6]. However, the latter was reported to occur in the mitochondrial intermembrane space isolated from rat liver [7,8] and yeast [9]. Subsequent studies in yeast revealed that only an immature form of Cu,Zn-superoxide dismutase, lacking both Cu$^{2+}$ and Zn$^{2+}$, could enter mitochondria. Moreover, the conserved disulphide bond in yeast Cu,Zn-superoxide dismutase, which is essential for its activity, was reduced [10].

The mitochondrial localization of Cu,Zn-superoxide dismutase suggests that the enzyme has a functional relationship with the occurrence of O$_2$$^•$− in this compartment. The concentration of O$_2$$^•$− in the intermembrane space would be determined by its vectorial release from the site O of the cytochrome bc$_1$ complex in the inner membrane [11] and by diffusion from cytosol across the mitochondrial outer membrane [12,13]. Hence, intermembrane space Cu,Zn-superoxide dismutase may be implicated not only in the regulation of redox-sensitive pathways within mitochondria, but also in maintaining steady-state levels of O$_2$$^•$− and H$_2$O$_2$ in the cytosol. However, it is not known whether post-translational modifications of Cu,Zn-superoxide dismutase (insertion of Zn$^{2+}$ and Cu$^{2+}$, and formation of conserved disulphide bond) take place in mammalian mitochondria in a similar fashion as in cytosol and whether intermembrane space Cu,Zn-superoxide dismutase is functional in intact mitochondria. Early studies reported that Cu,Zn-superoxide dismutase is in a latent state in intact mitochondria and becomes active after disruption of mitochondrial membranes [12–15].

The present study was aimed at assessing the functionality of intermembrane space Cu,Zn-superoxide dismutase and at elucidating the molecular basis for its latent state in intact mitochondria. These aims were addressed by modulating the redox status of Cu,Zn-superoxide dismutase and assessing its activity by spectroscopic methods.

EXPERIMENTAL

Chemicals

DMPO (5,5′-dimethyl-1-pyrroline-N-oxide), p-hydroxyphenylacetate, Cu,Zn-superoxide, bovine heart cytochrome c, partially

1 Abbreviations used: DMPO, 5,5′-dimethyl-1-pyrroline-N-oxide; IAM, iodoacetamide; NBT, Nitro Blue Tetrazolium; NEM, N-methylmaleimide; XTT, 3-(1-(phenylamino)-carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzenesulphonic acid hydrate.

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acetylated bovine heart cytochrome c, dextran sulphate (molecular mass 8000 Da), BSA, XTT (3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro]benzenesulphonic acid hydrate), digitonin, IAM (iodoacetamide), NEM (N-methylmaleimide) and uric acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). p-Nitrophenolate was from Aldrich Chemicals (Milwaukee, WI, U.S.A.).

Isolation of liver mitochondria

LIVER mitochondria were isolated from adult male Wistar rats by differential centrifugation as described previously [11]. Rat livers were excised, chopped into fine pieces, washed with 0.25 M sucrose and homogenized in isolation buffer containing 210 mM mannitol, 70 mM sucrose and 2 mM Hepes, pH 7.4, plus 0.05% (w/v) BSA. The homogenate was centrifuged at 800 g for 8 min, the pellet was removed, and the centrifugation process was repeated. The supernatant was centrifuged at 8000 g for 10 min, the pellet was washed with the isolation buffer, and the centrifugation was repeated. The pellet containing a mixture of organelles was further fractionated by centrifugation at 8500 g for 10 min in a Percoll gradient consisting of three layers of 18, 30 and 60% (w/v) Percoll in sucrose/Tris buffer (0.25 M sucrose, 1 mM EDTA and 50 mM Tris/HCl), pH 7.4) [16]. Mitochondria were collected from the interface of 30% and 60% Percoll and washed with the sucrose/Tris buffer. Mitochondrial proteins were determined as described in [17].

Isolation of mitochondrial intermembrane space contents

The mitochondrial outer membrane was disrupted selectively by treating mitochondria (40 mg/ml) with 0.11 mg of digitonin/mg of protein [18]. Mitoplasts were removed from the intermembrane space preparation by centrifugation at 10000 g for 15 min.

Assays of marker enzymes

The purity of isolated mitochondria was characterized enzymatically by measuring, in both granule- and Percoll-purified mitochondrial fractions, the activities of acid phosphatase (a lysosomal marker), urate oxidase (a peroxisomal marker), and fumarase and sulphite oxidase (mitochondrial markers), as described previously [19–22]. The activities of the marker enzymes in the mitochondrial fraction were expressed as percentages of total activities observed in the granule fraction.

Measurement of superoxide dismutase activity

Superoxide dismutase activity was assayed by generating O$_2^{-}$ with the xanthine/xanthine oxidase system in the presence of DMPO [11], acetylated cytochrome c [23] or sulphonated tetrazolium salt (XTT) [24]. Cyanide (5 mM) was used to inhibit selectively the activity of intermembrane space Cu,Zn-superoxide dismutase. Superoxide dismutase activity was also assayed in native gels following electrophoresis by NBT (Nitro Blue Tetrazolium) staining [25].

EPR spectroscopy

EPR spectra were obtained with a Bruker ECS 106 spectrometer (operating at X-band) equipped with a cylindrical cavity operating in TM$_{100}$ mode. Samples were transferred to bottom-sealed Pasteur pipettes (volume, 150 µl), and EPR spectra were obtained at room temperature (22 °C) with the following settings: microwave frequency, 9.77 GHz; microwave power, 20 mW; field modulation frequency, 100 kHz; field modulation amplitude, 2 G; receiver gain, 1×10$^6$; time constant, 164 ms; scan rate, 1.9 G·s$^{-1}$. The spectra shown were the result of four accumulations starting at 5 min after mixing the reaction components. Computer simulations of the spectra were obtained using the WinSIM program. DMPO, used in EPR experiments, was dissolved in water containing 0.1 mM DETAPAC (diethylenetriaminepentacetic acid) and was purified several times with activated charcoal; DMPO concentration was calculated spectrophotometrically ($ε_{232}$ = 7700 M$^{-1}$·cm$^{-1}$); the stock solution was kept under helium at −20 °C.

Fluorescence spectroscopy

Fluorescence measurements were performed on a PerkinElmer LS-5 spectrofluorometer. H$_2$O$_2$ formation was measured by monitoring horseradish-peroxidase-catalysed H$_2$O$_2$-dependent oxidation of p-hydroxyphenylacetate ($λ_{excitation}$ = 320 nm; $λ_{emission}$ = 400 nm) [26].

Modulation of redox state of mitochondrial thiol groups

Thiol groups of mitochondria were alkylated with either IAM [27] or NEM [28]. Mitochondria were incubated with 100 mM IAM in the presence of digitonin for 15 min. Intermembrane space content was then isolated by centrifugation, its pH was adjusted to 8.9, and incubated further at room temperature for 24 h. Alternatively, mitochondria (10 mg/ml) were incubated with 20 mM NEM for 1 h at room temperature in the presence of digitonin. In order to oxidize thiol groups, mitochondria (2 mg/ml) were treated with various concentrations of H$_2$O$_2$ for 5 min at 37 °C; the organelles were then washed and incubated with IAM (100 mM) in the presence of digitonin.

Table 1 Marker proteins and their activity in isolated rat liver mitochondria

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Granule fraction</td>
<td>Mitochondrial fraction</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Lysosomes</td>
<td>100</td>
</tr>
<tr>
<td>Urate oxidase</td>
<td>Peroxisomes</td>
<td>100</td>
</tr>
<tr>
<td>Fumarase</td>
<td>Mitochondrial matrix</td>
<td>100</td>
</tr>
<tr>
<td>Sulphite oxidase</td>
<td>Mitochondrial IMS</td>
<td>100</td>
</tr>
</tbody>
</table>

Data

Data are reported as means ± S.D. of at least three independent experiments.

RESULTS

Isolation and purity of mitochondrial preparations

The activities of marker enzymes for lysosomes, peroxisomes, and mitochondria were measured both in the granule- and Percoll-purified mitochondrial preparations. The activities of selected marker enzymes in mitochondrial preparations were expressed as percentages of total activity observed in the granule fraction before centrifugation in Percoll gradients (Table 1). The identity of isolated mitochondrial fractions was confirmed by the presence of high levels of fumarase and sulphite oxidase activities, located in mitochondrial matrix and intermembrane space respectively.
describe in the Experimental section. Carried out in sucrose/Tris buffer, pH 7.4. The DMPO–OH spin adduct was detected by EPR as mitochondrial (100 µM plus 5 mM KCN; (160 mM DMPO; (0.44 of DMPO carbon-centred radical (1:2:2:1; [29]. An EPR signal characteristic of the DMPO–OH spin adduct of the xanthine/xanthine oxidase system in the presence of DMPO, anion adduct (DMPO–OOH). Figure 1(D) presents the computer simulation of spectrum in Figure 1(C), assuming a mixture containing a 0.56 molar ratio of DMPO–OH (a= a= 14.9 G) and 0.44 of DMPO carbon-centred radical (a= 15.6 G; a= 22.6 G). The six-line spectrum, characteristic of a carbon-centred radical adduct with DMPO, is probably due to a DMPO–CN adduct [31]. Addition of intact mitochondria in either state 4 (Figure 1E) or state 3 (not shown) respiration did not affect EPR signal intensity, thus suggesting that Cu,Zn-superoxide dismutase in mitochondria does not scavenge externally generated O2•−. However, the DMPO–OH signal was abolished (Figure 1F) following the addition of a mitochondrial intermembrane preparation obtained by centrifugation of digitonin-treated mitochondria. Cyanide inhibited the O2•−-scavenging activity elicited by the mitochondrial intermembrane space preparation (Figure 1G).

In agreement with these findings, the rates of O2•− formation, monitored spectrophotometrically by following reduction of XTT or acetylated cytochrome c, were not affected by intact mitochondria (Table 2). The mitochondrial intermembrane preparation decreased the rate of reduction of XTT or acetylated cytochrome c. The O2•−-scavenging activity of the mitochondrial intermembrane space content was completely inhibited by cyanide (Table 2).

Considering that O2•− can diffuse across the mitochondrial outer membrane [11–13], these findings (Figure 1E and Table 2) suggest that the intermembrane Cu,Zn-superoxide dismutase is inactive in intact mitochondria, but it is activated following selective disruption of the outer membrane by digitonin (Figure 1F and Table 2). This notion was confirmed further by experiments addressing the dismutation of externally generated O2•− by mitochondria. O2•− generated by the xanthine/xanthine oxidase system partially dismutates to H2O2, which was detected by a peroxide-based assay (Figure 2, a). In addition, the xanthine/xanthine oxidase system is known to generate H2O2 directly [30], which contributes to the oxidation of p-hydroxyphenylacetate, the fluorescent probe in the peroxidase-based assay. Bovine Cu,Zn-superoxide dismutase converted all O2•− into H2O2, thereby greatly increasing the rate of p-hydroxyphenylacetate oxidation (Figure 2, b). Intact mitochondria did not alter the rate of H2O2 production (Figure 2, c), whereas the mitochondrial intermembrane preparation significantly increased H2O2 production (Figure 2, d). These findings strengthen the notion that loss of the mitochondrial outer membrane integrity is required for activation of Cu,Zn-superoxide dismutase.

Thiol groups play a key role in activation of intermembrane Cu,Zn-superoxide dismutase

Thiol–disulphide oxidoreductases catalyse thiol–disulphide exchange reactions that result in the formation or reduction of protein disulphide bonds. Protein disulphide-isomerase is a thiol–disulphide oxidoreductase that was recently shown to be located in mitochondrial outer membrane. Bacitracin (10 mM) [32], an inhibitor of protein disulphide-isomerase, considerably decreased

Table 2 Spectrophotometric analysis of O2•−-scavenging activity in intact mitochondria

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>XTT reduction (µM/min)</th>
<th>Acetylated cytochrome c reduction (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>0.57 ± 0.04</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.55 ± 0.04</td>
<td>n.d.</td>
</tr>
<tr>
<td>Intermembrane space</td>
<td>0.33 ± 0.03</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>Bovine Cu,Zn-SOD</td>
<td>0.005 ± 0.001</td>
<td>0.48 ± 0.04</td>
</tr>
</tbody>
</table>

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intermembrane space Cu,Zn-superoxide dismutase activity (60 ± 5 %) (Figure 3A) during digitonin treatment of mitochondria. This suggests that disulphide bond formation, catalysed at least in part by protein disulphide-isomerase, may be indeed the underlying basis for activation of intermembrane Cu,Zn-superoxide dismutase during disruption of the outer membrane by digitonin. Consistent with these findings, incubation of the mitochondrial intermembrane preparation with 4 mM dithiothreitol for 2 h at 37°C completely inactivated intermembrane space Cu,Zn-superoxide dismutase (results not shown).

Thiol alkylating agents, such as IAM or NEM, elicited two different effects on mitochondrial Cu,Zn-superoxide dismutase activity depending on the mitochondrial preparation: (i) treatment of intact mitochondria with alkylating agents in the presence of digitonin substantially decreased the activity of intermembrane space Cu,Zn-superoxide dismutase (Figure 3B); this is expected when considering that the enzyme occurs in the reduced (thiol moieties) state in intact mitochondria; (ii) treatment of mitochondrial intermembrane preparation with either NEM or IAM did not affect intermembrane space Cu,Zn-superoxide dismutase activity (Figure 3B), because the enzyme was already in its oxidized (disulphide bonds) state. These findings suggest that thiol groups are critical in the activation process of intermembrane space Cu,Zn-superoxide dismutase during disruption of the mitochondrial outer membrane, and that once intermembrane space Cu,Zn-superoxide dismutase is activated (thiol → disulphide transition), the thiol groups are no longer sensitive to the alkylating agents.

The role of the redox state of cysteine groups in the activation of intermembrane space superoxide dismutase was also assessed by examining its O$_{2}^{•−}$-scavenging activity in native polyacrylamide gels following electrophoresis by NBT staining. Intact mitochondria revealed a single band in inverted gels with an electrophoretic behaviour similar to that of Cu,Zn-superoxide dismutase (Figure 3C). The O$_{2}^{•−}$-scavenging activity of this band was inactivated completely by 5 mM KCN, thus indicating the Cu,Zn nature of superoxide dismutase (results not shown). Prior treatment of mitochondria with NEM or IAM substantially decreased the O$_{2}^{•−}$-scavenging activity of intermembrane space superoxide dismutase, thus supporting the critical role of thiol groups in the enzyme activation (Figure 3C). In IAM-treated mitochondria, however, several bands with residual O$_{2}^{•−}$-scavenging activity were observed; although the exact identity of these upper bands is unknown, they may be ascribed to intermembrane space Cu,Zn-superoxide dismutase with different extents of alkylation. Under non-denaturing conditions, the movement of proteins through the polyacrylamide gel matrix depends on a combination of their size, shape and charge. Cu,Zn-superoxide dismutase possesses three cysteine residues (6, 57 and 146), of which Cys$^{6}$ is in the reduced form and is not involved in superoxide dismutase activity. With a pK$_{a}$ of approx. 8.5, the thiol in Cys$^{6}$ is likely to be ionized, thereby contributing to the overall charge of Cu,Zn-superoxide dismutase. Different levels of alkylation of Cys$^{6}$ thiol in the superoxide dismutase homodimer may decrease the number of negative charges on the enzyme and thus slow its mobility in native electrophoresis. Therefore the change in the electrophoretic mobility of superoxide dismutase following treatment with IAM could be accounted for, in part, by changes in charge of the enzyme, rather than by its molecular mass or shape. In support of this notion, similar bands with substantially greater intensities were observed when intermembrane space superoxide dismutase
at a concentration of 100 µM into cytosol via voltage-dependent anion channels [4].

Figure 4 Effect of H$_2$O$_2$ on intermembrane space Cu,Zn-superoxide dismutase activation

(A) Mitochondria were incubated with the indicated concentrations of H$_2$O$_2$ for 5 min at 37°C, washed with sucrose/Tris buffer, pH 7.4, and incubated with digitonin in the presence of IAM for 1 h at room temperature. Cu,Zn-superoxide dismutase activity was assayed in the mitochondrial intermembrane space preparation by following the reduction of XTT at 470 nm, and was expressed as percentage of fully activated enzyme prepared by rupturing the outer membrane with digitonin alone. (B) Mitochondria were incubated with indicated concentrations of H$_2$O$_2$ for 5 min at 37°C. Intermembrane space Cu,Zn-superoxide dismutase activity was then measured spectrophotometrically in intact mitochondria following XTT reduction at 470 nm. Reactions were carried out in the sucrose/Tris buffer consisting of 20 mM-units/ml xanthine oxidase, 50 µM xanthine and 750 µM XTT. Where indicated, intact mitochondria were added at a concentration of 100 µg of protein/ml.

was treated with IAM after disruption of mitochondrial outer membrane.

H$_2$O$_2$ modulates the activity of intermembrane space Cu,Zn-superoxide dismutase

Pre-incubation of intact mitochondria with low micromolar concentrations of H$_2$O$_2$ prevented the inactivation of intermembrane space Cu,Zn-superoxide dismutase following disruption of the outer membrane in the presence of IAM (Figure 4A). Cyanide (5 mM) inhibited this activation, thus suggesting the Cu,Zn nature of the dismutase. A bell-shaped enzyme activation response to H$_2$O$_2$ concentrations was observed, with a maximum effect at 5 × 10$^{-6}$ M H$_2$O$_2$ (75 ± 6 %) (Figure 4A). It is noteworthy that treatment with H$_2$O$_2$ alone enabled intact mitochondria to scavenge externally generated superoxide (Figure 4B). It may be surmised that an oxidative modification of thiol groups of Cu,Zn-superoxide dismutase is required for its activation in the mitochondrial intermembrane space.

DISCUSSION

Recent studies confirmed the localization of Cu,Zn-superoxide dismutase in the mitochondrial intermembrane space of both rat liver [7,8] and yeast [9], and, thereby, ended a 30-year debate following the initial report on the presence of this enzyme in the intermembrane space [35]. The activity of mitochondrial superoxide dismutase is almost equally partitioned between the matrix (Mn-superoxide dismutase) and the intermembrane space (Cu,Zn-superoxide dismutase) [8,12,13] compartments, thus suggesting that the intermembrane space could be one of the major sites of exposure to O$_2$$^•−$. The mitochondrial inner membrane vectorially releases O$_2$$^•−$ into the intermembrane space [11] upon autoxidation of the ubisemiquinone at the side O of the cytochrome bc$_1$ complex; intermembrane space O$_2$$^•−$ can be released by mitochondria into cytosol via voltage-dependent anion channels [4]. Likewise, cytosolic O$_2$$^•−$ can diffuse across the outer membrane [4,12,13]. The NAD(P)H oxidase activity of the apoptosis-in-

Cu,Zn-superoxide dismutase is inactive in mitochondrial intermembrane space

Folding, metal insertion and thiol oxidation of cytosolic Cu,Zn-superoxide dismutase is a complex process that is only partially understood. Yeast Cu,Zn-superoxide dismutase is imported into yeast mitochondria in an immature form that lacks both Cu$^{2+}$ and Zn$^{2+}$ and possesses reduced cysteine residues [10]. While the mechanism for Zn$^{2+}$ insertion is not known, the copper chaperone for Cu,Zn-superoxide dismutase loads Cu$^{2+}$ into cytosolic Cu,Zn-superoxide dismutase [35]. Mechanistic studies demonstrated that the copper chaperone for Cu,Zn-superoxide dismutase is localized in yeast mitochondria where it plays a key role for efficient uptake of Cu$^{2+}$ and its insertion into nascent Cu,Zn-superoxide dismutase [10]. The biochemical pathways for oxidation of critical thiols of the intermembrane space Cu,Zn-superoxide dismutase are not known; however, it appears that thiol oxidation, rather than metal insertion, is the limiting step in the activation of Cu,Zn-superoxide dismutase, for the enzyme is inactive in intact mitochondria and is activated upon rupture of the mitochondrial outer membrane. The oxidation of Cys$^{37}$ and Cys$^{146}$, and formation of the intrasubunit disulphide bond is affected by the environmental redox status. These thiol moieties were not oxidized when eukaryotic Cu,Zn-superoxide dismutase was expressed in the prokaryotic cytosol, the latter being a more reducing environment than the former [36]. However, oxidation of critical thiols and activation of eukaryotic Cu,Zn-superoxide dismutase accompanied its export to the prokaryotic periplasmic space, which is a more oxidizing environment than the prokaryotic cytosol, or when it was expressed in the cytosol of prokaryotic cells lacking thioredoxin reductase [36].

Competitive kinetic studies (Figure 1 and Table 2) revealed that intact rat liver mitochondria neither scavenge nor dismutate externally generated O$_2$$^•−$. In agreement with earlier findings [12–15], disruption of the mitochondrial outer membrane led to recovery of the O$_2$$^•−$-scavenging (Figure 1 and Table 2) and -dismutating (Figure 2) activities, which were sensitive to cyanide, thus indicating the Cu,Zn nature of the dismutase. The activation of intermembrane space Cu,Zn-superoxide dismutase upon pre-treatment of intact mitochondria with H$_2$O$_2$ (Figure 4A) and its inactivation following treatment of mitochondria with alkylating agents during disruption of the outer membrane (Figure 3B) suggest that all post-translational modifications necessary for an active state of the enzyme have taken place in mitochondria, except formation of the intrasubunit disulphide bond. The steady-state levels of O$_2$$^•−$ and H$_2$O$_2$ in mitochondria and cytosol are similar at 10$^{-6}$ M and 10$^{-7}$ M respectively [37]. However, a more reducing mitochondrial environment, compared with the cytosol, might be the underlying reason for the inactive state of Cu,Zn-superoxide dismutase. Liver mitochondria possess both GSH/glutathione reductase and thioredoxin/thioredoxin reductase systems [38,39], which provide reducing equivalents to protect lipid membranes against oxidative damage, permeabilization and, in the case of the inner membrane, loss of the membrane potential. However, the overall redox potential of the mitochondrial
inter-membrane space has not been quantitatively compared with those of the mitochondrial matrix and the cytosol.

Mechanism of intermembrane space Cu,Zn-superoxide dismutase activation

Cellular enzymes known as thiol–disulphide oxidoreductases catalyse thiol–disulphide exchange reactions to promote the formation or reduction of protein disulphide bonds. The prototype of this group of enzymes is protein disulphide-isomerase [40]. Recently, protein disulphide-isomerase was demonstrated to be located in the mitochondrial outer membrane [41,42]. However, whether it resides in the cytosolic or intermembrane space side of the outer membrane is not known. Activation of intermembrane space Cu,Zn-superoxide dismutase upon disruption of outer mitochondrial membrane by digitonin is indicative of gain of access of intermembrane space Cu,Zn-superoxide dismutase to thiol–disulphide oxidoreductases that catalyse disulphide bond formation. Bacitracin [32], a well known inhibitor of protein disulphide-isomerase, partially inhibited intermembrane space Cu,Zn-superoxide dismutase activation following outer membrane disruption (Figure 3A), thus suggesting that protein disulphide-isomerase may constitute a significant part of the thiol-disulphide oxidoreductase activities that are possibly located in the cytosolic side of outer mitochondrial membrane. However, it remains to be elucidated whether or not thiol–disulphide oxidoreductases serve to activate intermembrane space Cu,Zn-superoxide dismutase following its release into the cytosol, as may be the case in, for example, the apoptotic disruption of mitochondrial outer membrane. Likewise, it remains to be assessed whether or not cytosolic activation of intermembrane space Cu,Zn-superoxide dismutase is implicated in the regulation of early events of programmed cell death.

The biochemical pathways that are implicated in the activation of Cu,Zn-superoxide dismutase in the intermembrane space are not known. Exposure of intact mitochondria to low concentrations (10^{-6} M) of H_2O_2 led to intermembrane space Cu,Zn-superoxide dismutase activation (Figure 4). These low peroxide levels suggest the involvement of an enzyme-mediated thiol oxidation rather than direct oxidation by H_2O_2. Potential candidates to catalyse such a reaction are thiol oxidases, such as phospholipid hydroperoxide glutathione peroxidase [43], and mitochondrial peroxiredoxins (III and/or V), a family of peroxidases that reduce H_2O_2 to water with the use of reducing equivalents provided by thiol-containing proteins [44–46]. H_2O_2 may serve as a substrate for phospholipid hydroperoxide glutathione peroxidase located on the inner mitochondrial membrane, where it may catalyse the oxidation of intermembrane space Cu,Zn-superoxide dismutase. However, it is not known whether the K_M of this enzyme for H_2O_2 is sufficiently low to function at low micromolar concentrations of this substrate. Mitochondrial peroxiredoxins have a very low K_M (~2 M) for H_2O_2, and are inactivated by hyperoxidation of their catalytic cysteine residues upon exposure to higher concentrations of H_2O_2 [47]. Exposure of HeLa or A549 cells to 100 M H_2O_2 for 10 min reversibly inactivated mitochondrial peroxiredoxins [48]. This feature of mitochondrial peroxiredoxins may also explain the observed bell-shaped enzyme activation response to H_2O_2 (Figure 4A), where concentrations higher than 10 M were less effective in activating intermembrane space Cu,Zn-superoxide dismutase, probably due to a hyperoxidative inactivation of mitochondrial peroxiredoxins. Because the thioredoxin reductase system catalyses the reduction of protein disulphide bonds [49], the activity of intermembrane space Cu,Zn-superoxide dismutase may be regulated through redox changes inherent in the thiol–disulphide conversion as an intermediate in the mitochondrial peroxiredoxin/thioredoxin reductase system. Although peroxiredoxin III and V, and thioredoxin reductase reside in mitochondria, their submitochondrial localization is not known. It is an intriguing possibility that the reversible activation of Cu,Zn-superoxide dismutase in the intermembrane space may function to divert O_2^{**} from its reaction with cytochrome c to form a more diffusible oxidant, H_2O_2, which can act as a redox signal both in mitochondria and cytosol.

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