Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore

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Opening of the mitochondrial permeability transition pore (MPTP) is sensitized to [Ca^{2+}] by oxidative stress (diamide) and phenylarsine oxide (PAO). We have proposed that both agents cross-link two thiol groups on the adenine nucleotide translocase (ANT) involved in ADP and cyclophilin-D (CyP-D) binding. Here, we demonstrate that blocking Cys^{160} with 80 μM eosin 5-maleimide (EMA) or 500 μM *N*-ethylmaleimide (NEM) greatly decreased ADP inhibition of the MPTP. The ability of diamide, but not PAO, to block ADP inhibition of the MPTP was antagonized by treatment of mitochondria with 50 μM NEM to alkylate matrix glutathione. Binding of detergent-solubilized ANT to a PAO-affinity matrix was prevented by pre-treatment of mitochondria with diamide, EMA or PAO, but not NEM. EMA binding to the ANT in submitochondrial particles (SMPs) was prevented by pre-treatment of mitochondria with either PAO or diamide, implying that both agents modify Cys^{160}. Diamide and PAO pre-treatments also inhibited binding of solubilized ANT to a glutathione S-transferase–CyP-D affinity column, both effects being blocked by 100 μM EMA. Intermolecular cross-linking of adjacent ANT molecules via Cys^{57} by copper phenanthroline treatment of SMPs was abolished by pre-treatment of mitochondria with diamide and PAO, but not with EMA. Our data suggest that PAO and diamide cause intramolecular cross-linking between Cys^{160} and Cys^{257} directly (not antagonized by 50 μM NEM) or using glutathione (antagonized by 50 μM NEM) respectively. This cross-linking stabilizes the 'c' conformation of the ANT, reducing the reactivity of Cys^{57}, while enhancing CyP-D binding to the ANT and antagonizing ADP binding. The two effects together greatly sensitize the MPTP to [Ca^{2+}].

Key words: apoptosis, cell death, cyclophilin D, diamide, oxidative stress, phenylarsine oxide.

INTRODUCTION

The mitochondrial permeability transition pore (MPTP) is a non-specific channel present in the inner mitochondrial membrane that opens under conditions of elevated matrix [Ca^{2+}]. Several factors greatly enhance the sensitivity of the pore to [Ca^{2+}], of which the most potent and relevant to the cellular setting are oxidative stress, adenine nucleotide depletion and increased inorganic phosphate concentration. Opening of the MPTP has two major consequences. First, by permeabilizing the inner membrane to any molecule of less than approx. 1.5 kDa, ion and metabolite gradients across the inner membrane are dissipated, leading to a collapse of the protonmotive force. This converts the mitochondria from net producers of ATP into net users as the proton-translocating ATPase reverses. Secondly, when the MPTP opens there is an equilibration of all small solutes across the membrane, leaving a colloidal osmotic pressure exerted by the matrix proteins that causes massive swelling of the mitochondria and outer-membrane rupture [1–3]. The MPTP normally remains closed, but can open under conditions associated with oxidative stress and elevated intracellular [Ca^{2+}]. Two such situations are exposure to chemical toxins and during reperfusion following a prolonged period of ischaemia (reperfusion injury). Indeed, it is thought that the opening of the MPTP may be a critical factor in determining both the extent of cell death and the pathways taken under such conditions. Extensive opening will lead to necrotic cell death, since the loss of ATP will lead to major ionic disturbances and activation of degradative enzymes that compromise normal cell function. However, transient opening will induce apoptosis, since ATP concentrations can be maintained, but mitochondrial swelling may be sufficient to release pro-apoptotic intermembrane proteins such as cytochrome *c*, apoptosis-inducing factor ('AIF') and DIABLO (direct IAP binding protein with low pI) [2,4–7].

As a result of extensive studies from this and other laboratories, it is widely held that the MPTP is formed via an interaction of mitochondrial cyclophilin-D (CyP-D) with the adenine nucleotide translocase (ANT) [2,4–6]. Some workers have proposed that the voltage-activated anion channel ('VDAC') of the outer membrane may also be involved [2,5,6], although our own data do not support this view [4,8]. CyP-D exhibits peptidylprolyl *cis–trans* isomerase activity [9,10], which suggests that its role is to induce a conformational change in the ANT. Cyclosporin A (CsA), a potent inhibitor of the MPTP, binds to CyP-D and prevents its interaction with the ANT [8]. In contrast, adenine nucleotides inhibit the MPTP by binding to both high- and low-affinity sites of the ANT, preventing calcium from inducing the CyP-D-mediated conformational change [11]. Stimulation of the MPTP may occur with carboxyatractyloside (CAT), a potent inhibitor of the ANT, which is thought to act by preventing adenine nucleotide binding to the high-affinity site [11]. Oxidative stress greatly increases the sensitivity of the MPTP to [Ca^{2+}] [11,12] by two mechanisms; first, by decreasing adenine nucleotide binding to both high- and low-affinity sites, and secondly by

Abbreviations used: ANT, adenine nucleotide translocase; BKA, bongkrekate; CAT, carboxyatractyloside; CsA, cyclosporin A; CyP-D, cyclophilin D; DTT, dithiothreitol; EMA, eosin 5-maleimide; MPTP, mitochondrial permeability transition pore; NEM, *N*-ethylmaleimide; PAO, phenylarsine oxide; PEG, poly(ethylene glycol); SMP, submitochondrial particle.

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increasing CyP-D binding [11,13]. We have proposed that oxidation of critical thiol groups on the ANT may be responsible for these effects, and have suggested that these residues may be Cys^{57} (CyP-D binding) and Cys^{160} (adenine nucleotide binding) [11]. (Note that the numbering of the cysteine residues here is on the basis of the rat sequence, as opposed to the bovine sequence used previously, with residues 57, 160 and 257 referred to here corresponding to residues 56, 159 and 256 in the bovine sequence.) This suggestion is compatible with the data of Bernardi and co-workers [14–16], which has implicated two thiol groups in MPTP opening. These can be selectively oxidized by different protocols and show different sensitivity to the reagents monobromobimane and *N*-ethylmaleimide (NEM). At low concentrations (25–50 μM), both reagents preferentially react with GSH in intact mitochondria, and prevent it from being oxidized. This inhibits the activation of the MPTP by diamide and t-butylhydroperoxide, implying a role of GSSG in the action of these agents on the MPTP, most probably via thiol cross-linking [15]. In the present study, we utilize a range of thiol reagents and oxidative protocols that differentially target critical matrix thiol groups of the ANT. Our data provide evidence that both diamide and the vicinal thiol reagent phenylarsine oxide (PAO) both stimulate MPTP opening by cross-linking Cys^{257} with Cys^{160}. Selective modification of Cys^{160} with eosin 5-maleimide (EMA) confirms the importance of this residue for the inhibition of the MPTP by adenine nucleotides.

EXPERIMENTAL

Materials

Mitochondria were isolated from livers or hearts of 250 g male Wistar rats and purified by Percoll density-gradient centrifugation, as described previously [9]. Submitochondrial particles (SMPs) and purified inner-mitochondrial membranes were prepared from mitochondria, as described previously [8,17] with the addition of protease inhibitors (4 mg/ml pepstatin A, leupeptin, antipain and 0.5 mM PMSF and benzamidine) to the isolation media. Protein concentrations were determined by the Bradford assay.

Antibodies

Polyclonal antibodies against purified recombinant rat CyP-D and the C-terminus of ANT were raised in rabbits as described previously [8], whereas an additional polyclonal antibody against rat heart mitochondrial ANT purified as described previously [8] was raised in rabbits by a standard procedure [18]. The sources of all other reagents are as described previously [8,11].

Methods

Assay of MPTP opening

The sensitivity of the MPTP to [Ca^{2+}] and [ADP] was determined by measuring the rate of shrinkage of pre-swollen mitochondria upon addition of poly(ethylene glycol) (PEG), as described previously [11]. Mitochondria were swollen by incubating for 20 min at 30 °C and 2 mg of protein/ml under de-energized conditions in KSCN buffer [150 mM KSCN/20 mM Mops/10 mM Tris/0.5 μM rotenone/0.5 μM antimycin (pH 7.2)] containing 1 mM $CaCl_2$. Additions of diamide, PAO or NEM to the buffer were made as required. Swelling was terminated by addition of 1.2 mM EGTA and centrifugation at 12000 ***g*** for 10 min to sediment the swollen mitochondria, which were then resuspended at 20 mg protein/ml in KSCN buffer containing 2 mM nitrilotriacetate and 2 μM A23187. Shrinkage of these swollen mitochondria at different Ca^{2+} and ADP concentrations following addition of 50 % (w/v) PEG 2000 (0.5 ml added to 3 ml of mitochondrial suspension) was constantly monitored by measurement of A_{520} (10 data points/s).

Binding of the ANT to a glutathione S-transferase (GST)–CyP-D affinity column

This was performed essentially as described previously [8]. GSH–Sepharose B [50 μl of a 50 % (w/v) slurry; Amersham Biosciences) was washed in KP_i buffer [20 mM KH_2PO_4/0.1 mM EDTA (pH 7.2)], and the beads were collected by centrifugation at 700 ***g*** for 30 s, before incubating with 125 μg of GST–CyP-D for 15 min at room temperature, followed by washing. When required, the bound GST–CyP-D was incubated with 25 μM CsA for 15 min at room temperature. Inner mitochondrial membranes from mitochondria pre-treated with thiol reagents, as required, were partially solubilized at 1 mg of protein/ml in KP_i buffer containing 0.5 % (w/v) Triton X-100, and 1 ml of this preparation was mixed with the immobilized GST–CyP-D for 30 min at room temperature. The Sepharose beads were washed five times with 1 ml of KP_i buffer containing 0.5 % (w/v) Triton X-100, followed by 1 ml of KP_i buffer containing 3 % (w/v) Triton X-100 and then 1 ml of KP_i buffer + 0.5 % (w/v) Triton X-100 and 500 mM NaCl. The specifically bound protein was then eluted by incubation for 30 min with 75 μl of elution buffer [75 mM Tris/HCl, pH 8.0, containing 300 mM NaCl, 10 mM GSH and 0.5 % (w/v) Triton X-100] and analysed by SDS/PAGE and Western blotting, as described previously [8,19].

Binding of proteins to a PAO-affinity column

Synthesis of a PAO-affinity column and its binding of ANT were performed essentially as described previously [11]. Mitochondria, pre-treated with thiol reagents or ANT ligands as required, were solubilized at 10 mg/ml in 50 mM Hepes, pH 7.2, containing 150 mM Na_2S0_4, 1 mM EDTA, 3 % (w/v) Triton X-100, 4 mg/ml pepstatin A, leupeptin, antipain and 0.5 mM each of PMSF and benzamidine. Insoluble matter was pelleted by centrifugation at 125000 ***g*** for 10 min at 4 °C. Solubilized mitochondria (600 μl) were passed through a 0.5 ml PAO column twice. The column was washed with 20 column-vols. of solubilization buffer containing 0.25 % (w/v) Triton X-100. Bound protein was eluted with solubilization buffer containing 0.25 % (w/v) Triton X-100 and 10 mM dithiothreitol (DTT), and then analysed by SDS/PAGE and Western blotting.

Binding of recombinant CyP-D to SMPs

SMPs prepared from control and appropriately treated mitochondria (see the Figure legends for further details) were suspended at 4 mg/ml in isolation buffer and incubated for 15 min at room temperature with 25 μM CsA or an equal volume of solvent (ethanol). Recombinant CyP-D, produced by thrombin cleavage of GST–CyP-D [8], was pre-incubated with 50 μM CsA or an equal volume of ethanol (solvent) for 15 min at room temperature, and then 10 μg was incubated with 100 μg of SMPs for 15 min at room temperature. After sedimentation by centrifugation (160000 ***g*** for 5 min at 4 °C), SMPs were washed once in isolation buffer.

Fluorescent labelling of mitochondrial proteins with EMA

SMPs derived from control and pre-treated mitochondria were labelled with EMA as outlined by Majima et al. [20]. SMPs were incubated with 100 μM EMA at a concentration of 10 mg

protein/ml for 30 s on ice in the dark. The reaction was stopped by the addition of 50 mM DTT, and proteins were separated by SDS/PAGE. Labelled proteins were visualized using a UV transilluminator.

RESULTS AND DISCUSSION

Effects of thiol reagents on the sensitivity of the MPTP to inhibition by ADP

We have shown previously that treatment of mitochondria with either diamide or PAO greatly impairs the ability of ADP to inhibit the MPTP [11]. These reagents both cross-link adjacent thiol groups, and it is known that the ANT contains three cysteine residues on the matrix surface: Cys^{57}, Cys^{160} and Cys^{257} [20,21]. Since EMA, which behaves as an adenine nucleotide analogue and preferentially attacks Cys^{160} of the ANT [21,22], also prevented ADP inhibition of the MPTP, it seemed probable that both diamide and PAO modified this thiol group. However, which of the adjacent thiol groups provides the cross-linking partner remains unclear [11]. In order to identify the relevant cysteine residues, we first investigated the effects of NEM alone, or in combination with diamide or PAO, on inhibition of the MPTP by ADP. Intact mitochondria were incubated with NEM (50 μM) at 0 °C for 10 min, before further treatment with diamide or PAO. Under these conditions, the major effect of such low concentrations of NEM is to react with glutathione and prevent glutathione-mediated thiol cross-linking [15]. It should be noted that the pre-treatment with 50 μM NEM was performed at 5 mg of protein/ml, i.e. 10 nmol/mg of protein, and thus not much in excess of the glutathione content of mitochondria (4–7 nmol/mg of protein) [16]. Thus the majority of the reagent added would be expected to react with GSH. In contrast, with pre-swollen mitochondria or SMPs that contain no GSH, such concentrations of NEM preferentially attack Cys^{57} with higher concentrations, also modifying Cys^{160} [21]. It should be noted that even in intact mitochondria some modification of Cys^{57} may occur at 50 μM NEM, but the extent of this is not great, as will be discussed further below when the effects of copper phenanthroline are described.

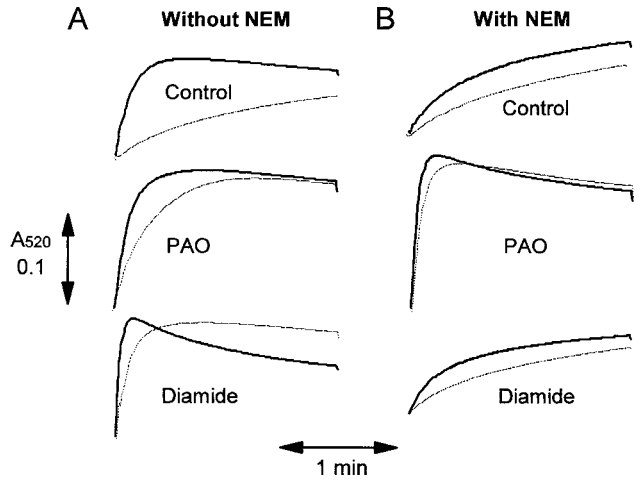

Figure 1 Effect of NEM on the ability of diamide and PAO treatment of mitochondria to activate the MPTP

Mitochondria (5 mg/ml in isolation buffer) were incubated at 0 °C in isolation buffer for 10 min in the presence or absence of 50 μM NEM, and then sedimented by centrifugation before resuspending at 2 mg/ml in KSCN swelling buffer (see the Experimental section) in the presence or absence of 20 μM PAO or 100 μM diamide, as indicated. Swelling was initiated by the addition of 1 mM $CaCl_2$ and incubation at 30 °C for 20 min, before termination by addition of 1.5 mM EGTA and collection of mitochondria by centrifugation. The extent of MPTP opening was determined after addition of 40 μM buffered Ca^{2+} and, when required, 50 μM ADP (shown by the dotted lines) using the PEG shrinkage technique (see the Experimental section).

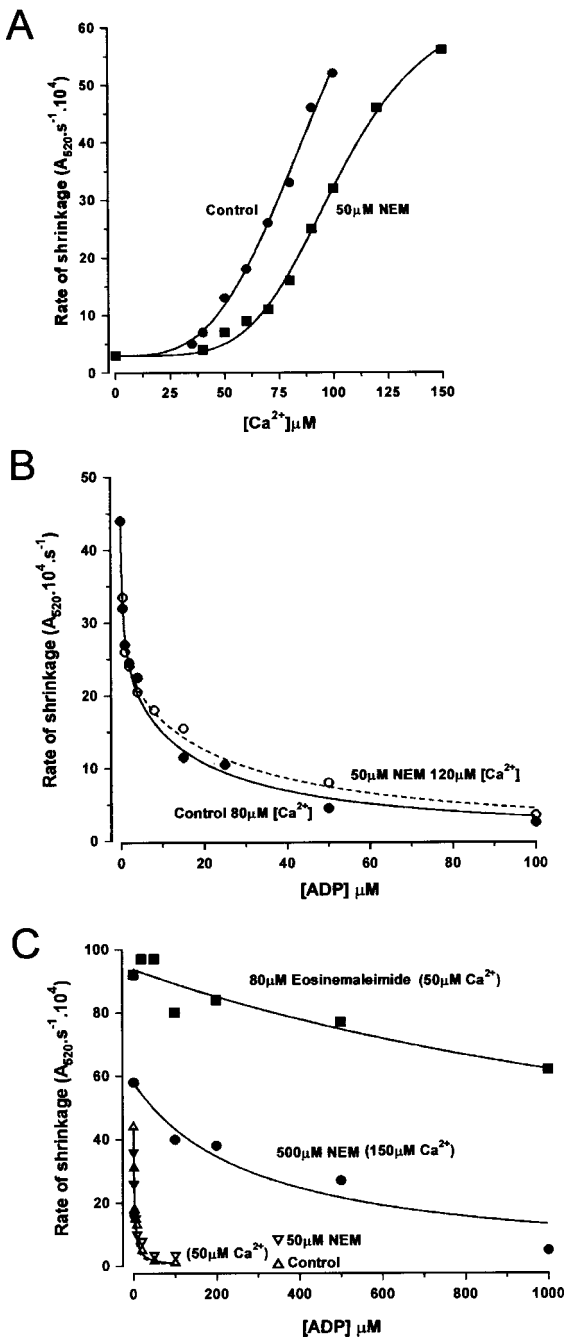

Figure 2 Effect of different thiol reagents on the calcium- and ADP-sensitivity of the MPTP

Mitochondria were pre-swollen in KSCN buffer for 18 min, as described in the legend to Figure 1, before addition of 50 μM NEM, 500 μM NEM or 80 μM EMA, as indicated. After a further 2 min, mitochondria were treated with EGTA, collected by centrifugation, and MPTP activity was assayed, as described for Figure 1. Rates of swelling were calculated by differentiation of the traces, as described previously [12].

In Figures 1 and 2, the shrinking of pre-swollen mitochondria induced by PEG to assess the extent of MPTP opening is shown. The data in Figure 1(A) confirm that treatment of mitochondria with either 100 μM diamide or 20 μM PAO during the initial swelling increased MPTP opening at a Ca^{2+} concentration of 40 μM, and greatly decreased the ability of ADP to inhibit pore opening. In Figure 1(B), parallel data are presented for mitochondria pre-treated with 50 μM NEM. Some reduction in pore opening was observed for mitochondria not exposed to either diamide or PAO, which may reflect some modification of Cys^{57} by NEM under these conditions. However, more dramatic was the ability of NEM treatment to abolish the effect of diamide, while having no effect on the stimulation by PAO. The modest effect of pre-treatment with 50 μM NEM alone was found to reflect a decrease in sensitivity to [Ca^{2+}], but not [ADP], as shown in Figures 2(A) and 2(B). In contrast, pre-treatment with 500 μM NEM decreased further the sensitivity of the MPTP to Ca^{2+}, perhaps reflecting greater modification of Cys^{57}, but in this case the more dramatic effect was to greatly decrease the ability of ADP to inhibit the MPTP, in a manner analogous to that of EMA. Taken together, these data allow two tentative conclusions to be drawn. First, when 50 μM NEM is used to alkylate glutathione, and possibly also some Cys^{57} residues, the major effect in the absence of diamide or PAO is a modest reduction in the calcium-sensitivity of the pore. In contrast, modification of Cys^{160} with 500 μM NEM or 80 μM EMA activates the MPTP by preventing inhibition by ADP, in agreement with our previous data [11]. Secondly, the ability of low concentrations of NEM to prevent activation of the MPTP by diamide but not PAO implies that diamide cross-links two thiol groups of the ANT in a glutathione-dependent manner, whereas PAO may chemically bridge the same residues independently of glutathione. These thiol groups are unlikely to include Cys^{57}, and are thus more likely to be Cys^{160} and Cys^{257}.

Effect of different thiol reagents on the binding of ANT to a PAO-affinity column

We have shown previously that the detergent-solubilized ANT can bind to a PAO-affinity column [11]. In Figure 3, the effects of the thiol reagents used above on this binding are shown. As expected, pre-incubation of mitochondria with 0.1 mM PAO totally blocked ANT binding, whereas pre-incubation of mitochondria with diamide (1 mM) greatly decreased binding (Figure 3B). In contrast, NEM at 50 μM had no significant effect on binding, although 0.5 mM NEM slightly decreased binding (Figure 3B), suggesting that, at this concentration, NEM may also attack Cys^{160}, as has been observed in SMPs [21]. Consistent with this, studies by Costantini et al. [23] have demonstrated that treatment of mitochondria with still higher concentrations of NEM (1 mM) in the presence of calcium can inhibit binding of the ANT to a PAO column to a similar extent as that of diamide treatment. In order to study the effects of EMA, which is not freely permeable across the inner membrane, this reagent was added after opening the MPTP by addition of 350 μM Ca^{2+} for 7 min. At a concentration of 100 μM, this reagent, unlike NEM, did cause a significant decrease in ANT binding to the PAO-affinity column (Figure 3C). Pre-treatment of mitochondria with CAT or bongkrekate (BKA) (Figure 3D) were without effect on binding. Since CAT treatment induces the 'c' conformation of the ANT, in which Cys^{57} is unreactive towards a variety of thiol reagents [21,24–26], these data confirm that binding of the ANT to PAO does not involve Cys^{57}. We have also demonstrated that pre-treatment of mitochondria with either CAT or BKA does not prevent the activation of MPTP opening induced by either

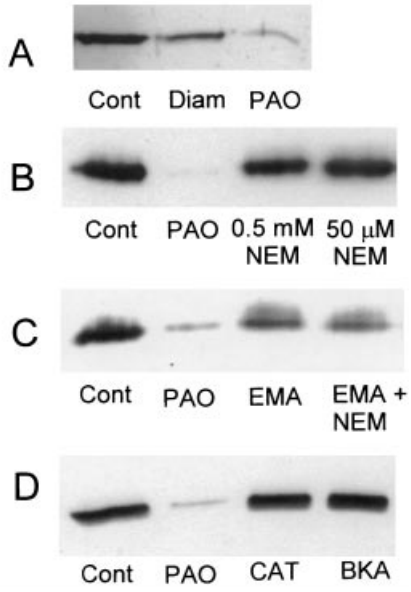

Figure 3 Effect of different thiol reagents and ANT ligands on the binding of ANT to a PAO-affinity column

In (**A**), (**B**) and (**D**), mitochondria (5 mg/ml) were treated with 50 or 500 μM NEM for 10 min on ice or 1 mM diamide, 0.1 mM PAO, 20 μM CAT or 20 μM BKA for 10 min at 22 °C, before solubilization in Triton X-100. Extracts were then passed through a PAO-affinity column, followed by extensive washing and elution of bound protein with DTT, as described in the Experimental section. Proteins were separated by SDS/PAGE and ANT was revealed by Western blotting. In (**C**), prior to detergent solubilization, mitochondria were pre-swollen by incubating at 10 mg of protein/ml in swelling buffer (see the Experimental section) at 25 °C for 5 min, before addition of 3 mM succinate and 350 μM $CaCl_2$ and incubation for a further 7 min [35]. Where required, incubation with EMA (100 μM) was then performed in the dark at 0 °C for 30 s, and the reaction was terminated by addition of 1 mM DTT. Mitochondria were then resealed by the addition of 2 mM EGTA and washed twice in swelling buffer, before resuspending in twice the volume of swelling buffer and treatment with 50 μM NEM or 0.1 mM PAO as required. Cont, control.

diamide or PAO, again eliminating Cys^{57} as a target for the action of these agents (results not shown).

EMA is fluorescent, and thus, following SDS/PAGE, it was possible to visualize proteins that had reacted with this reagent by viewing the gel under UV light. This is illustrated in Figure 4. Although many fluorescent bands are visible, and the labelling of several was decreased in SMPs derived from diamide- and PAO-treated mitochondria, there is a major band at 30 kDa that corresponds to the position of the ANT, as revealed by Western blotting. Both diamide and PAO treatment greatly decreased the labelling of this band by EMA. Thus the data of Figures 3 and 4 support the conclusions of the experiments reported in Figures 1 and 2, that PAO and diamide must cross-link Cys^{160} with another thiol group. Since our data have effectively eliminated Cys^{57} as a candidate, this thiol group is most likely to be Cys^{257}.

Effect of different thiol reagents on the binding of ANT to a GST–CyP-D-affinity column

Previous studies from our laboratory [8] and that of Crompton and co-workers [27] have demonstrated that detergent-solubilized ANT binds specifically to a GST–CyP-D affinity column. In Figure 5, we present data on the effects of pre-treating mitochondria with the different thiol reagents on such binding. Figure 5(A) shows that both diamide and PAO treatment increased ANT binding in a manner that was partially prevented by pre-

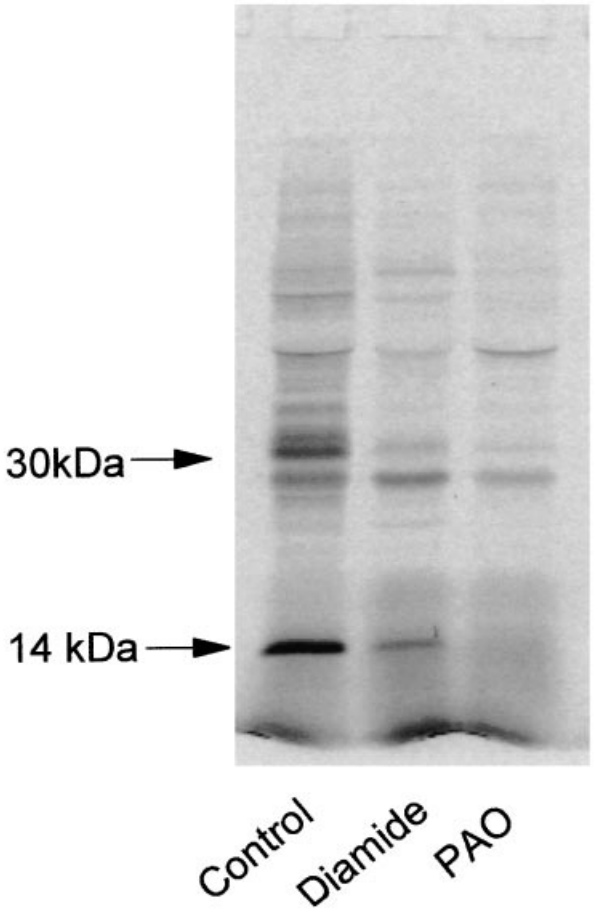

Figure 4 Effect of diamide and PAO on the labelling of inner mitochondrial membrane proteins with EMA

Submitochondrial particles were prepared from rat liver mitochondria after incubation in the presence or absence of 1 mM diamide or 0.1 mM PAO for 10 min at 22 °C, as described in the legend to Figure 3. The SMPs (10 mg/ml) were then incubated with 100 μM EMA for 30 s at 0 °C in the dark. The reaction was stopped by addition of 50 mM DTT, followed by an equal volume of SDS/PAGE sample buffer. Following separation of protein by SDS/PAGE, EMA-labelled proteins were visualized by UV transillumination.

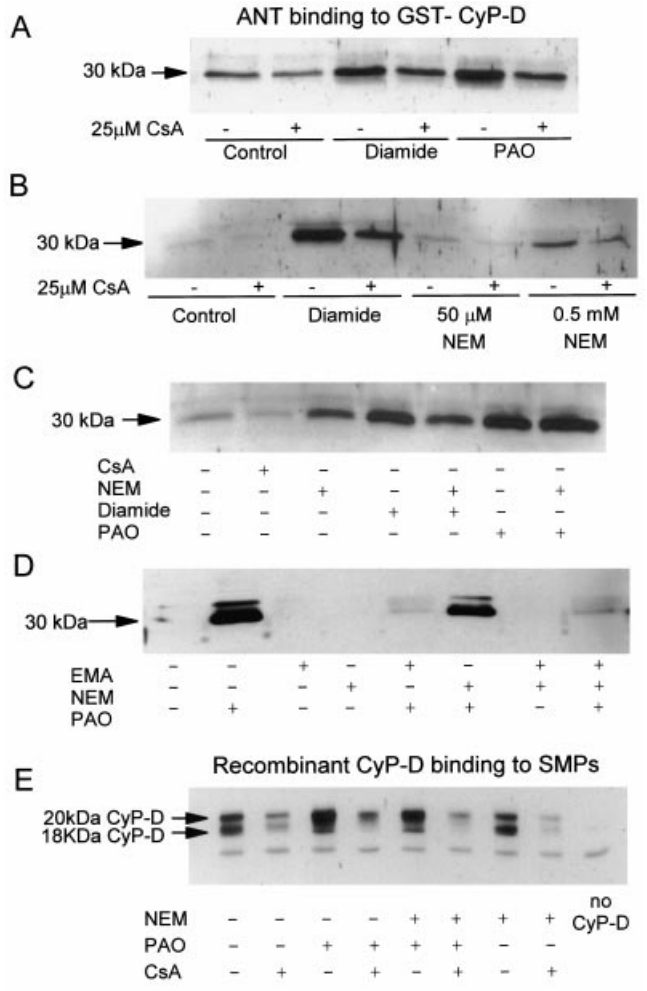

Figure 5 Effects of different thiol reagents on the binding of ANT to CyP-D-affinity column and GST–CyP-D binding to SMPs

(**A**–**D**) Mitochondria were pre-treated with PAO (0.1 mM), diamide (1 mM), NEM at 50 μM {(**B**), (**C**) and (**D**) [and also at 0.5 mM in (**B**)]} or EMA (100 μM; **D**) as described in the legend to Figure 3, before preparation of inner mitochondrial membranes and their solubilization and passage through a GST–CyP-D affinity column, as described in the Experimental section. After elution of bound proteins with GSH, samples were separated by SDS/PAGE and the ANT was revealed by Western blotting. Where indicated, the GST–CyP-D column was pre-incubated with 25 μM CsA before addition of the solubilized inner mitochondrial membranes that also contained 25 μM CsA. (**E**) SMPs were prepared from mitochondria treated with 0.1 mM PAO in the presence and absence of 50 μM NEM, as above. Binding of recombinant CyP-D was determined as described in the Experimental section.

exposure of the GST–Cyp-D column to CsA. In contrast, 50 μM NEM had no effect on binding, whereas 500 μM NEM produced a small increase (Figure 5B). Although the extent to which ANT from control membranes bound to the GST–CyP-D column varied between experiments (compare Figures 5A and 5B), the increase in binding induced by diamide and PAO was always substantial. Furthermore, when control binding was larger (Figure 5A), it also demonstrated a larger CsA-insensitive component, suggesting this may be non-specific binding of denatured ANT to the GST–CyP-D column. It is known that detergent-solubilized ANT is unstable, and denatures readily [28]. Pre-treatment of mitochondria with 50 μM NEM before exposure to diamide decreased ANT binding to the GST–CyP-D column, whereas it had no effect on the increase in binding induced by PAO treatment (Figures 5C and 5D). However, treatment of pre-swollen mitochondria with 100 μM EMA prior to membrane solubilization (see above) did abolish the increase in binding caused by PAO treatment. In order to confirm that the effects of PAO on ANT binding to the GST–CyP-D column did not merely reflect greater denaturation of the detergent-solubilized ANT by this reagent, the experiment of Figure 5(C) was performed. Here, recombinant CyP-D was added to SMPs that had been prepared from control mitochondria and those pre-treated with 50 μM NEM and 0.1 mM PAO, either alone or in combination. These data confirm that, in the absence of detergent, PAO treatment increases CyP-D binding to the SMPs, but once again the effect is not prevented by pre-treatment with 50 μM NEM. Taken together, the data of Figure 5, like those derived from the effects of thiol reagents on MPTP activity (Figures 1 and 2) and ANT binding to a PAO-affinity column (Figure 3), are consistent with diamide and PAO cross-linking Cys^{160} with Cys^{257}, the former reagent requiring glutathione to exert its effects.

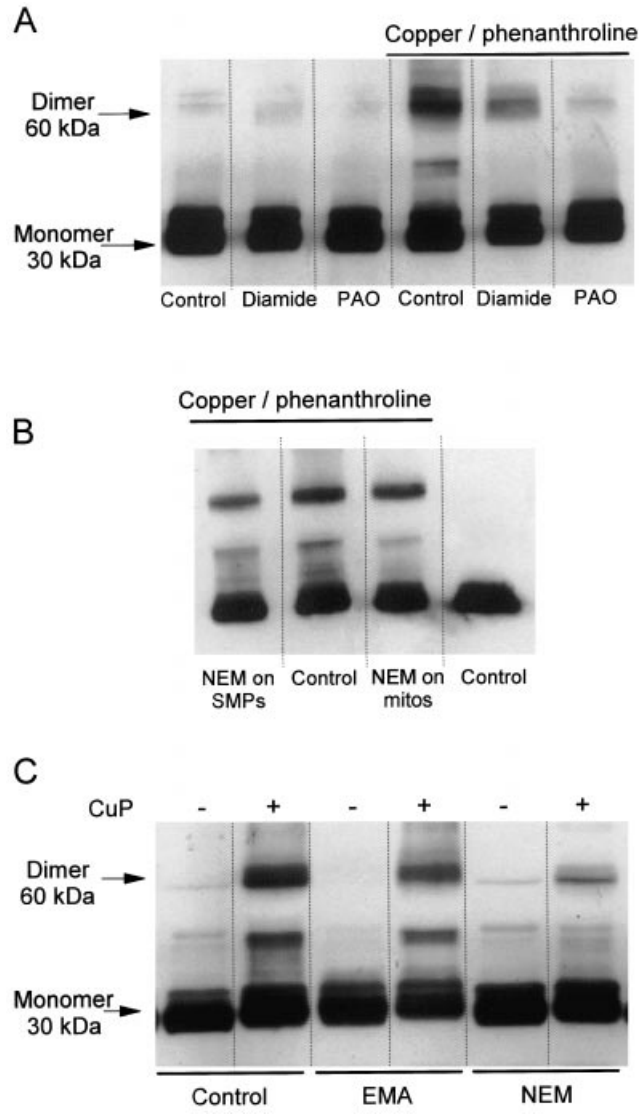

Figure 6 Effect of different thiol reagents on the dimerization of ANT by copper *o*-phenanthroline (CuP)

(**A**) Mitochondria were pre-treated with 1 mM diamide or 0.1 mM PAO as described in the legend to Figure 3, before preparation of SMPs, whereas in (**B**), either the isolated mitochondria were pre-treated with 50 μM NEM prior to SMP preparation, as in Figure 1 or the SMPs themselves were treated with 50 μM NEM for 30 s at 0 °C in SMP preparation buffer, followed by washing. (**C**) Control SMPs were incubated in the presence or absence of either 80 μM EMA or 0.4 mM NEM for 30 s at 0 °C in SMP preparation buffer, followed by washing. Where indicated, SMPs (4 mg/ml in preparation buffer) were treated with 0.1 mM CuP for 10 min at 0 °C to induce ANT dimerization and the was reaction stopped with 5 mM EDTA. Proteins (20 μg) were separated by SDS/PAGE under non-reducing conditions, and the ANT was revealed by Western blotting.

Effect of different thiol reagents on the dimerization of the ANT by copper phenanthroline

In order to exclude further an involvement of Cys^{57} in the effects of PAO and diamide, we made use of the ability of copper phenanthroline to cross-link two ANT monomers in SMPs via their Cys^{57} residues to produce a covalent dimer. This is detectable as a 60 kDa band on non-reducing SDS/PAGE, as shown in Figure 6, with an additional band at approx. 40 kDa probably representing the dimer of a proteolytic breakdown product of the

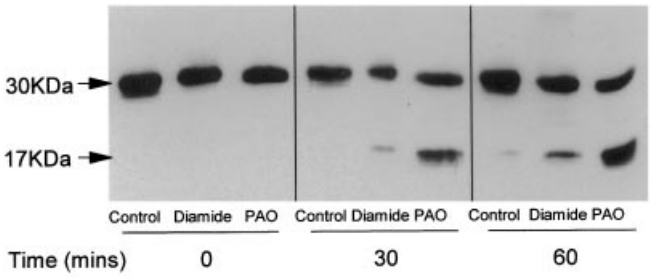

Figure 7 Effect of diamide and PAO treatment of mitochondria on the sensitivity of the ANT to cleavage by endogenous proteases

Mitochondria were suspended in isolation buffer at 5 mg of protein/ml and solubilized by addition of an equal volume of buffer containing 40 mM KH_2PO_4, 40 mM KCl, 2 mM EDTA and 6% (w/v) Triton X-100 at pH 6.0. Samples were taken immediately, or allowed to stand at room temperature (22 °C) for 30 min and 60 min, as indicated, before subjecting to SDS/PAGE and Western blotting with anti-ANT antibodies.

ANT (see below). It should be noted that demonstration of the presence of the dimerized ANT required greater protein loading of the SDS/PAGE prior to Western blotting. This revealed a second minor immunoreactive band, with slightly lower mobility than the major 30 kDa band that represents ANT2, the major ANT isoform in liver mitochondria. The most likely identity of this second band is ANT1, a minor isoform in liver, but the major isoform in heart mitochondria. Our antibody detects both isoforms with equal sensitivity (results not shown). Dimerization of the ANT was greatly inhibited by addition of 50 or 400 μM NEM to the SMPs prior to the copper phenanthroline in order to block Cys^{57} (Figures 6B and 6C). However, pre-treatment of mitochondria with 50 μM NEM to alkylate glutathione before SMP formation did not greatly inhibit cross-linking by copper phenanthroline (Figure 6B), confirming little modification of Cys^{57} under these conditions. Treatment of SMPs with 80 μM EMA, which preferentially attacks Cys^{160}, had much less effect on dimerization than NEM, as predicted (Figure 6C). In contrast, when SMPs were prepared from mitochondria pre-treated with 0.1 mM PAO or 1 mM diamide, exposure to copper *o*-phenanthroline no longer induced Cys^{57} dimerization of the ANT (Figure 6C), implying that Cys^{57} was no longer accessible to this reagent. Neither PAO nor diamide treatment induced Cys^{57} dimerization of the ANT in their own right (Figure 6A). At first glance, these data might appear to contradict our conclusion that PAO and diamide cross-link Cys^{159} with Cys^{257}, rather than attack Cys^{57}. However, Cys^{57} is known to be unreactive towards thiol reagents when the ANT is in the 'c' conformation [21,24–26], and thus an alternative explanation would be that when Cys^{159} is cross-linked with Cys^{257} the ANT takes up a conformation similar to the 'c' conformation, in which Cys^{57} is no longer reactive. This is consistent with the observation that CAT, like both diamide and PAO, decreases the affinity of the MPTP for ADP, whereas BKA is without effect [11]. It should be noted that there are high- and low-affinity binding sites for adenine nucleotides that inhibit pore opening, both of which are blocked by diamide and PAO treatment, whereas only the higher affinity site is blocked by CAT [11].

Further evidence that both diamide and PAO treatment induce a conformational change in the ANT is provided in Figure 7. Here, we present data on the effects of incubating detergent-solubilized mitochondria, in the absence of protease inhibitors, on the appearance of 17 kDa proteolytic degradation products of

Table 1 Summary of the effects of various thiol reagents and ANT ligands on MPTP function

Degrees of stimulation and inhibition are indicated by the number of '+' and '−' signs given. 'No effect' is indicated by 0, whereas 'ND' indicates 'not determined'.

	Mitochondria treated with ...							Mitochondria pre-treated with 50 μM NEM on ice prior to swelling		
Function	NEM (50 μM)	NEM (500 μM)	EMA (80 μM)	PAO (0.1 mM)	Diamide (1 mM)	CAT (20 μM)	BKA (20 μM)	Control	PAO (0.1 mM)	Diamide (1 mM)
Kinetics										
Initial rate of shrinkage	−	−−	++	++	+++	+*	0*	−	+++	0
Inhibition by ADP	0	−−	−−−	−−−	−−	−−*	0*	0	−−−	0
Activation by Ca^{2+}	−	−−	ND	++	++	+*	0*	ND	ND	ND
ANT binding to GST–CyP-D	0	+	0	+++	++	−†	−†	0/+	++	0
ANT binding to PAO column	0	−	−−	−−−	−−	0	0	0	ND	ND
Binding of CyP-D to SMPs	0	ND	ND	++	ND	ND	ND	0	+	ND
Copper phenanthroline cross-linking of ANT	ND	−−−	−	−−−	−−	ND	ND	0	ND	ND

* Data are taken from [11].
† Data are from [8].

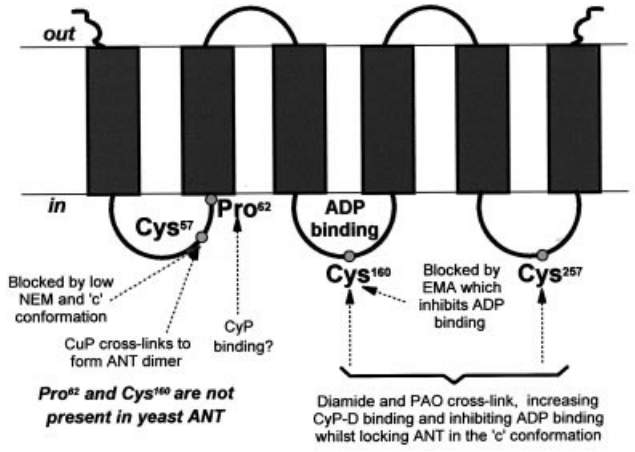

Figure 8 Summary diagram to illustrate the proposed role of ANT thiol groups in MPTP opening

the ANT. It can be seen that pre-treatment with either diamide or PAO before solubilization increased the susceptibility of the ANT to degradation by endogenous proteases. These data imply that both diamide and PAO treatment have induced a conformation of the ANT that is more sensitive to proteolysis.

Conclusions

The data we present in this paper are summarized in Table 1, and are consistent with oxidative stress, such as that induced by diamide and the vicinal thiol reagent PAO internally cross-linking the ANT between Cys^{160} and Cys^{257}. Our conclusions, summarized schematically in Figure 8, are consistent with the previous data of Torok and Joshi [29]. These authors demonstrated that treatment of the partially purified ANT with 0.1 mM copper phenanthroline at 23 °C (as opposed to 50 mM at 0 °C on SMPs, as used here) can cause intramolecular cross-linking of Cys^{160} and Cys^{257}, confirming the closeness of these residues. More recently, Majima et al. [30] have demonstrated that, in Triton-solubilized ANT, copper phenanthroline can also cross link Cys^{57} and Cys^{257}, but this does not occur when the ANT is in its normal membrane environment. In the case of diamide, cross-linking of Cys^{160} and Cys^{257} probably involved GSSG, since it is prevented by low concentrations of NEM that alkylate glutathione, making it unavailable for use in thiol oxidation. This accounts for the ability of NEM to inhibit MPTP opening, as observed by others [15]. Since Cys^{160} is located in the matrix adenine nucleotide-binding site of the ANT [31], both diamide and PAO, like EMA, greatly sensitize the MPTP to $[Ca^{2+}]$ by decreasing adenine nucleotide binding to the ANT (Figures 1 and 2). Both reagents also increase the binding of the solubilized ANT to CyP-D (Figure 5), which we have proposed may involve Pro^{62} of the ANT, in view of the peptidylprolyl *cis–trans* isomerase activity of CyP-D [9]. This proline residue is close to Cys^{57}, whose ability to form an intermolecular cross-link with Cys^{57} on an adjacent ANT is prevented by both diamide treatment and PAO treatment (Figure 6A). This suggests that the conformation of the ANT around Cys^{57} and Pro^{62} may change following cross-linking of Cys^{160} and Cys^{257}, and so account for the increase in CyP-D binding. A conformational change is confirmed by the increased sensitivity of the ANT to endogenous proteolytic cleavage following diamide and PAO treatment (Figure 7). Furthermore, Pro^{62} is absent in the three ANT isoforms found in yeast mitochondria, which do not exhibit a CsA-sensitive MPTP [11].

Our data provide no support for covalent dimerization of two ANT molecules via a Cys^{57} cross-link having a role in the opening of the MPTP. This is at variance with the conclusions of Brenner and co-workers [23], who have presented data that suggest an important role for Cys^{57} in MPTP opening. However, our data fit well with conclusions reached by Bernardi and co-workers [14–16], who have also provided data to suggest that two distinct thiol groups are implicated in modulating MPTP activity. One is sensitive to oxidation of GSH, e.g. by t-butylhydroperoxide or diamide, and is protected by both monobromobimane and NEM. These agents react with GSH, preventing it from being oxidized and catalysing disulphide formation between adjacent thiol groups. This site is likely to represent the cross-linking of Cys^{160} with Cys^{257}. Another thiol group on the MPTP was identified by Bernardi and co-workers [15], which responds to the redox state of matrix NAD(P), and is also blocked by NEM, but not by monobromobimane. It is proposed that this

site accounts for the well-documented stimulatory effect of oxidation of matrix NADH on the MPTP, perhaps through the mediation of thioredoxin or lipoamide [32,33]. This could also be due to modification of Cys^{57} by a non-glutathione-mediated mechanism, with NEM exerting a direct effect by blocking Cys^{57}. An additional level at which redox-sensitive regulation of the MPTP might be exerted is the mechanism by which the disulphide bonds formed between Cys^{160} and Cys^{257} are reduced again. This process has been shown to involve thioredoxin reductase and glutathione reductase, whose activities can be inhibited by 5,5′-dithiobis-(2-nitrobenzoic acid) in parallel with its activation of MPTP opening [34].

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