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Induction of the mitochondrial permeability transition by *N*-ethylmaleimide depends on secondary oxidation of critical thiol groups. Potentiation by copper-*ortho*-phenanthroline without dimerization of the adenine nucleotide translocase

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Abstract

Addition to energized rat liver mitochondria of low micromolar concentrations of the thiol oxidant, copper-*o*-phenanthroline [$Cu(OP)_2$], causes opening of the permeability transition pore, a cyclosporin A-sensitive channel. The effects of $Cu(OP)_2$ can be reversed by reduction with dithiothreitol (DTT), suggesting that a dithiol-disulfide interconversion is involved. However, at variance with all pore inducers known to act through dithiol oxidation, the effects of $Cu(OP)_2$ are not prevented by treatment of mitochondria with low (10–20 μM) concentrations of *N*-ethylmaleimide (NEM). Rather, these concentrations of NEM potentiate the inducing effects of $Cu(OP)_2$. We show that this enhancing effect of NEM is blocked by the subsequent addition of DTT, indicating that potentiation by NEM is mediated by an oxidative event rather than by substitution as such. We find that also pore induction by high (0.5–1.0 mM) concentrations of NEM in the absence of oxidants is completely blocked by reduction with DTT or β-mercaptoethanol. These results underscore the unexpected importance of oxidative events in pore opening by substituting agents. Since we find that pore opening by $Cu(OP)_2$ or by high concentrations of NEM is not accompanied by dimerization of the adenine nucleotide translocase, we conclude that the translocase itself is not the target of the pore-inducing oxidative events triggered by $Cu(OP)_2$ and NEM.

Keywords: Mitochondrion; Channel; Membrane permeability; Cyclosporin A; Oxidative stress

1. Introduction

The permeability transition (PT) is an abrupt permeability increase of the inner mitochondrial membrane which can be observed after matrix Ca^{2+} accumulation [1]. The PT can be favored by a large

Abbreviations: PT, permeability transition; MTP, mitochondrial permeability transition pore; CsA, cyclosporin A; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; MBM, monobromobimane; $Cu(OP)_2$, copper-*o*-phenanthroline; β-ME, β-mercaptoethanol; SMP, submitochondrial particles; $TPMP^+$, triphenylmethylphosphonium ion; HAP, hydroxylapatite; MOPS, 4-morpholinopropane sulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CAPS, 3-cyclohexylamino-1-propane sulfonic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; EGTA, ethylene-bis(oxoethylenenitrilo) tetraacetic acid

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series of heterogeneous compounds and conditions, the so-called inducers [2] acting on a complex channel, the CsA-sensitive MTP. Recent work indicates that the MTP is regulated, and that inducers converge on one or more discrete regulatory step(s) [3]. A key feature of the system is control by the proton electrochemical gradient, whereby the pore is modulated by both the transmembrane electrical potential difference (depolarization favoring pore opening [4,5]), and matrix pH (acidification favoring pore closure through critical histidyl residues [6]). The probability of pore opening can be modified either by changes of the membrane potential, or by changes of the threshold potential at which pore opening occurs through discrete sites (see [7,8] for reviews within this framework).

In recent years, our laboratory has addressed the issue of the role of SH groups in regulation of the PT. We have shown that the MTP is affected by dithiol-disulfide interconversions at a critical site (dubbed the 'S site'); that the disulfide is associated with a higher probability of pore opening [9–11]; and that the actual S site oxidant appears to be matrix GSSG [12]. Several agents (e.g. organic hydroperoxides, redox-cycling compounds, dithiol oxidants like diamide, and dithiol crosslinkers like phenylarsine oxide) act through this site, and their inducing effects can be blocked either by reduction with DTT, or by treatment with low concentrations (10–20 μM) of *N*-ethylmaleimide (NEM) [9], monobromobimane (MBM) [10] or benzoquinone [13]. In the course of a screening of oxidants for their effects on the PT, we found that the pore can be opened by low micromolar concentrations of the dithiol oxidant copper-*o*-phenanthroline ($Cu(OP)_2$) through an effect which could be reversed by DTT. Unexpectedly, however, this effect of $Cu(OP)_2$ was potentiated rather than inhibited by 25 μM NEM, and this potentiation was completely blocked by the subsequent addition of DTT. These findings suggested that potentiation by NEM is also mediated by an oxidative event rather than by substitution as such; and provided a working hypothesis for the well-known inducing effects of high (0.5–1.0 mM) concentrations of NEM in the absence of oxidants [14–17]. We find that pore induction by NEM and other substituted maleimides is also completely blocked by reduction with dithiothreitol (DTT) or β-mercaptoethanol (β-ME). While treatment of beef heart submitochondrial particles (SMP) with $Cu(OP)_2$ induces the formation of disulfide bridges at Cys^{56} on adjacent molecules of the adenine nucleotide translocase (ANT) [18], we find that pore opening by $Cu(OP)_2$ (or high NEM) is not accompanied by dimerization of this protein. Taken together, these results (i) underscore the unexpected importance of oxidative events in the PT induced by substituting agents of pathophysiological relevance; (ii) identify SH groups distinct from the S site as the target of $Cu(OP)_2$ and of NEM; and (iii) indicate that the effects of $Cu(OP)_2$ and high NEM on the PT are not related to dimerization of the ANT. This work has been presented in abstract form [19].

2. Materials and methods

Liver mitochondria from albino Wistar rats weighing about 300 g were prepared by standard differential centrifugation as described previously [20]. MTP opening was followed as the change of absorbance or of 90° light scattering at 540 nm with a Perkin Elmer LS-50 Spectrofluorimeter. Mitochondria (0.5 mg/ml) were incubated in thermostatted, magnetically stirred cuvettes (final volume 2 ml), and pore opening was triggered after accumulation of a limited Ca^{2+} load as specified in the figure legends. In Figs. 2 and 4 the data are expressed as the fractional pore opening (Φ) calculated as described in detail elsewhere [9]. Incubation media and further details are specified in the figure legends.

Mitochondrial membrane potential in the presence of 1 μM cyclosporin A (CsA) (to prevent depolarization following pore opening) was calculated from the distribution of triphenylmethylphosphonium ion ($TPMP^+$) with a $TPMP^+$-selective electrode exactly as described previously [9]. We found that the agents used in this study (NEM and $Cu(OP)_2$, alone or in combination) did not significantly alter the resting membrane potential or its response to uncoupler (not shown, but see [9,11] for representative titrations).

In the experiments of Fig. 5, rat liver mitochondria were suspended in 0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate-Tris, 1 mM Pi, 2 μM rotenone, 0.5 μg/ml oligomycin, and 10 μM

EGTA-Tris at a protein concentration of 0.5 mg/ml (final volume 40 ml). The additions specified in the figure legend were made to individual incubations, and the absorbance at 540 nm was checked on 2-ml aliquots. After 10 min (a time point at which, depending on the treatment, the pore was fully open or still closed, see legend to Fig. 5) mitochondria were sedimented by centrifugation at $10\,500\times g$, and each pellet was extracted on ice with 1 ml of 0.5 M NaCl, 0.5 mM EDTA-Tris, 10 mM Tris-MOPS pH 7.2, 5% Triton X-100 for 10 min. Partial purification of the ANT was then performed by hydroxylapatite (HAP) chromatography.

The extracts were incubated with HAP (Bio-Rad) which had been sequentially washed three times with 10 mM NaPi, pH 6.8 and twice with extraction buffer without Triton X-100 (1.5 ml of HAP was used for each sample). After 10–15 min of incubation at 4°C under gentle agitation, the samples were centrifuged for 10 min at $2600\times g$, the supernatants were carefully decanted, and insoluble material was sedimented by centrifugation with an ALC 4224 microfuge operating at top speed, and held at 4°C. To remove Triton X-100, the cleared supernatants were precipitated with trichloroacetic acid or cold acetone as described [21]. The precipitated proteins were solubilized with double strength Laemmli's gel sample buffer [22] under nonreducing conditions for 30 min at room temperature, and samples were resolved by SDS-PAGE according to Laemmli [22] in 12% acrylamide-0.3% bisacrylamide slab gels. The gels were electroblotted onto nitrocellulose sheets in 10 mM CAPS-NaOH pH 11, 10% MetOH (overnight transfer at 4°C, 2 mA/cm^2). The membrane was stained with Ponceau red (Sigma, St. Louis, MO), photographed, destained and finally immunodecorated with a rabbit antibody against the C-terminus of bovine ANT (a generous gift of Prof. G. Brandolin, Grenoble, France), which cross-reacts with the rat liver protein (see Fig. 5B). The primary antibody was used at a 1:1000 dilution, and reaction was revealed with a secondary peroxidase-conjugated anti-rabbit antibody (Sigma, St. Louis, MO).

SMP from bovine hearts were prepared and treated with $Cu(OP)_2$ exactly as described [18,23]. Extraction, partial purification of the ANT and Western blotting were performed as for mitochondria, except that 5 mg of protein and 0.7 ml of HAP were used for each incubation.

All chemicals were of the highest purity commercially available.

3. Results

In our effort to rationalize the effects of SH group reagents (oxidants and others) on the MTP, we have carried out a screening to determine the spectrum of compounds able to interact with the S site, a critical dithiol (e.g. [9–12]). The experiments of Fig. 1 illustrate the results we obtained when the dithiol oxidant, $Cu(OP)_2$, was used. Mitochondria energized with succinate in a sucrose-based, Pi-containing medium were loaded with a small Ca^{2+} pulse, which was unable to induce pore opening per se (not

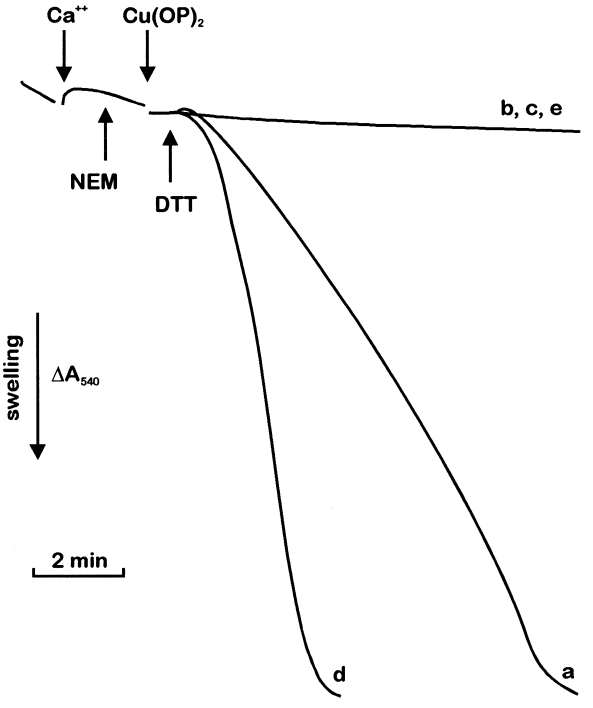

Fig. 1. Effects of NEM, DTT, and CsA on spontaneous MTP opening by $Cu(OP)_2$. The incubation medium contained 0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate-Tris, 1 mM Pi, 2 μM rotenone, 0.5 μg/ml oligomycin, and 10 μM EGTA-Tris. Final volume 2 ml, 25°C. The experiments were started by the addition of 1 mg of mitochondria (not shown). Where indicated by the arrows 5 μM Ca^{2+}, 25 μM NEM (traces d and e), 2.5 μM $Cu(OP)_2$ and 1 mM DTT (traces b and e) were added. In trace c, mitochondria were preincubated with 0.85 μM cyclosporin A.

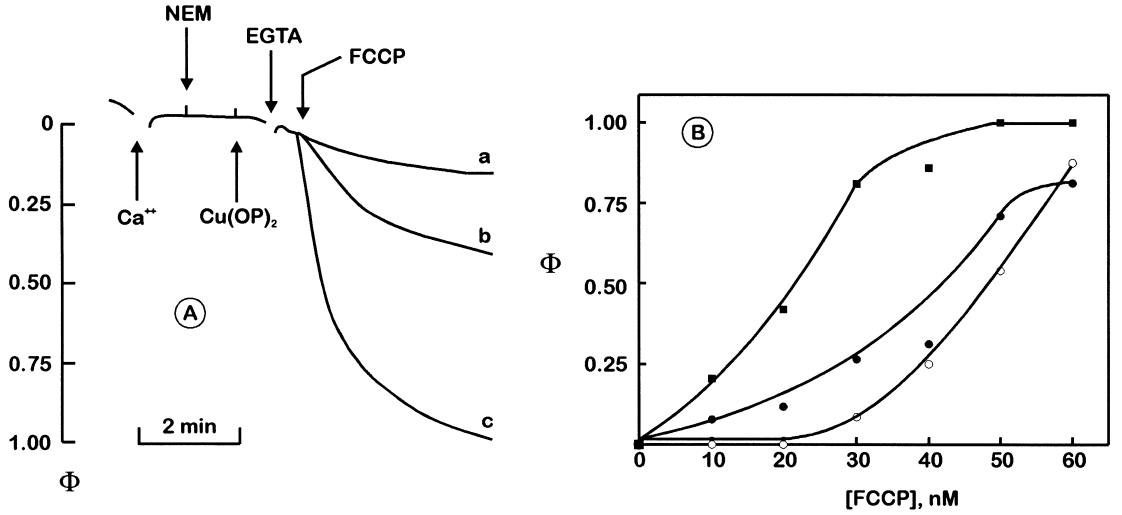

Fig. 2. Effects of $Cu(OP)_2$ and NEM on the fraction of mitochondria permeabilized by FCCP. Experimental conditions were exactly as in Fig. 1. (A) Where indicated, 5 μM Ca^{2+}, 25 μM NEM (trace c), 2.5 μM $Cu(OP)_2$ (traces b and c), 0.5 mM EGTA-Tris and 30 nM FCCP were added. (B) The experiments were carried out exactly as shown in panel A. The indicated concentrations of FCCP were added after Ca^{2+} alone (open circles), or after treatment with 2.5 μM $Cu(OP)_2$ (closed circles) or with both 2.5 μM $Cu(OP)_2$ and 25 μM NEM (closed squares). Φ denotes the fraction of mitochondria permeabilized to sucrose by FCCP.

shown). Addition of 2.5 μM $Cu(OP)_2$ was followed, after a short lag phase, by a process of absorbance decrease linked to sucrose permeation (trace a). Permeabilization by $Cu(OP)_2$ was effectively blocked by DTT (trace b), suggesting that the expected dithiol oxidation was taking place, and by CsA (trace c), implicating the MTP in the permeabilization process. Quite unexpectedly, however, pore opening was *favored* rather than inhibited by the addition of 25 μM NEM (trace d), and potentiation by NEM (like permeabilization by $Cu(OP)_2$) could be completely prevented by reduction with DTT added *after* NEM (trace e). It should be remembered (i) that this concentration of NEM fully blocks the S site, and thus prevents the effects of all inducers interacting with this site, including dithiol oxidants like diamide [9]; and (ii) that 25 μM per se does not cause pore opening in these protocols [9].

The inducing effect of $Cu(OP)_2$ and its potentiation by NEM are quantitatively analyzed in the experiments of Fig. 2. In these protocols, mitochondria were allowed to accumulate a small Ca^{2+} load, and the MTP status was then probed with the addition of a small concentration of the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) (30 nM in this experiment). Under basal conditions pore opening occurred in a negligible fraction of mitochondria (panel A, trace a). Upon the addition of $Cu(OP)_2$, however, the same concentration of FCCP caused opening of the pore in a larger fraction of mitochondria (panel A, trace b), and this effect was amplified by pretreatment with NEM (panel A, trace c). In Fig. 2B, the results are shown as a function of the FCCP concentration. It can be appreciated that the combination of NEM and $Cu(OP)_2$ was extremely effective at inducing pore opening for small depolarizations, suggesting that the threshold voltage for pore opening had been shifted to more negative values, i.e. closer to the resting potential.

The potentiating effect of NEM (a substituting agent) on the pore-inducing effects of $Cu(OP)_2$ (an oxidant) offered a clue about a potential mechanism by which NEM itself, at high concentrations, could be causing its well known effects as a pore inducer [14]. The experiment of Fig. 3 shows that the addition of 0.5 mM NEM to Ca^{2+}-preloaded mitochondria caused the expected permeabilization to sucrose after a lag phase (trace a). Permeabilization was fully sensitive to CsA (trace b) but not to 25 μM NEM (trace c) or MBM (not shown) indicating that the MTP was not opening through an effect at the S site. The novel result of Fig. 3 is that addition of DTT *after* NEM, but prior to onset of the PT, was able to block the PT (trace d). Furthermore, DTT was able to stop further mitochondria from undergoing the PT even when added after the permeabili-

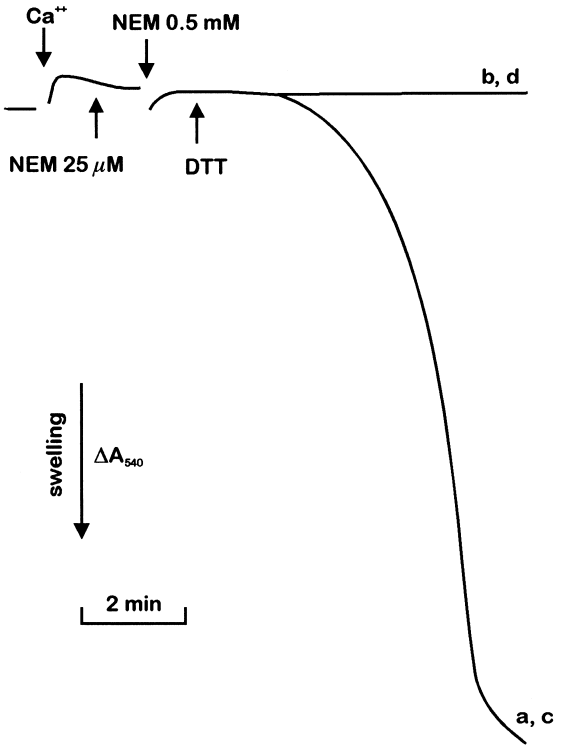

Fig. 3. Effect of DTT and CsA on mitochondrial permeabilization by high concentrations of NEM. Experimental conditions were as in Fig. 1. Where indicated 5 μM Ca^{2+}, 25 μM NEM (trace c), 0.5 mM NEM and 1 mM DTT (trace d) were added. In trace b, mitochondria were preincubated with 0.85 μM CsA.

zation process had begun (not shown). Similar results were obtained if β-ME was added instead of DTT in protocols based on uncoupler titrations analogous to those described in Fig. 2. Fig. 4 indeed shows that in the absence of NEM the addition of 30 nM FCCP was not followed by pore opening (panel A, trace a), while treatment with 1 mM NEM allowed pore opening in nearly all mitochondria (panel A, trace b). The effect of NEM was largely prevented by subsequent treatment with 1 mM β-ME (panel A, trace c). The shift in the responding population is documented most dramatically in Fig. 4B. In the presence of 1 mM NEM nearly all mitochondria opened the pore(s) already at 30 nM FCCP, a concentration unable to cause pore opening either in control incubations, or in NEM-treated mitochondria reduced with β-ME (Fig. 4B).

$Cu(OP)_2$ is able to induce both intra- and intermolecular crosslinking in the ANT under specific conditions [18,23–26]. Due to the long-standing suggestion that the ANT itself may be part of the MTP, we decided to test if indeed the effects of $Cu(OP)_2$, NEM and S site reagents on MTP were accompanied by modifications of the ANT which could be referred to formation of intermolecular disulfide bridges.

To this purpose, succinate-energized rat liver mitochondria were incubated in sucrose medium and supplemented with a 10 μM Ca^{2+} pulse, a condition which was not accompanied by pore opening, as assessed by light scattering measurements. Parallel incubations of mitochondria were additionally treated with 2.5 μM $Cu(OP)_2$, 25 μM NEM followed by 2.5 μM $Cu(OP)_2$, 1 mM NEM, 1 mM arsenite, 10 μM *tert*-butylhydroperoxide, or 0.1 mM diamide, all conditions that were accompanied by pore opening, again assessed by light scattering measurements. Mitochondria from all incubations were then sedimented by centrifugation, the ANT was partially purified by HAP chromatography, and analyzed by SDS-PAGE followed by transfer onto nitrocellulose and Western blotting with an antibody against the C-terminus of the ANT (Fig. 5A,B). It can be clearly appreciated that under these experimental conditions the ANT was present as the monomer (m, arrow), while no evidence of dimer formation was obtained except for a faint band with diamide (d, arrow, lanes 7). As expected [18,23–26] ANT dimer formation readily followed treatment of beef heart SMP with $Cu(OP)_2$ (lanes 9), and this was inhibited by NEM (lanes 10). Note that in these experiments the concentration of $Cu(OP)_2$ was increased to 100 μM since 2.5 μM was not able to induce ANT crosslinking even in SMPs (not shown).

4. Discussion

4.1. Mechanism of induction of the PT by NEM

In essence, the results presented in this paper demonstrate that induction of the mitochondrial PT by NEM is not due to substitution as such, but rather to the secondary oxidation of a cryptic site (or sites) causing in turn an increased probability of pore opening. Based on the inhibitory effects of DTT and, particularly, of β-ME on MTP opening induced by NEM we conclude that these oxidizable sites are thiol groups on cysteinyl residues, and that they are not generally accessible to oxidation unless a primary

site has been derivatized with NEM or other substituted maleimides. The location of the NEM-reactive site(s) remains undefined. It is noteworthy, however, that the relative potency at pore induction correlates with the octanol-water partition coefficient of substituted maleimides, since the more lipophilic *N*-benzylmaleimide and *N*-butylmaleimide are more effective pore inducers than NEM on a molar basis [16,17]. This finding suggests that the reactive sites may be embedded within the membrane's lipid phase. The NEM-reactive site(s) could either be part of an integral membrane protein (possibly the pore itself) or be located on pore regulatory proteins (such as cyclophilin D). The primary NEM-reactive site is probably a thiol as well, since the PT caused by other SH reagents like methylmethanethiosulfonate and mersalyl could also be fully inhibited by DTT and β-ME (data not shown).

In summary, these data make it possible to rationalize pore opening by substituting agents as a two-step event. The first step would be binding to the primary reactive SH group(s). This, in turn, would then cause a change in conformation unmasking a secondary, oxidizable site (probably a thiol group) mediating the second step, i.e. an oxidation leading to an increased probability of pore opening. This scheme is strongly supported by the finding that the PT is fully inhibited by adding a reductant (DTT or β-ME) *after* NEM, proving that oxidation occurs downstream of the NEM reaction step in the pore-activating sequence. The putative oxidizable dithiol is distinct from the previously identified S site [9–12] since the former cannot be blocked by MBM and, obviously, by NEM (see also the next section).

4.2. Mechanism of induction of the PT by $Cu(OP)_2$

A second relevant observation is that the MTP can be induced by the membrane impermeant thiol oxidant, $Cu(OP)_2$, and that this effect is inhibited by DTT (Figs. 1 and 2). At variance with other PT inducers acting through dithiol oxidation (like diamide, menadione and organic hydroperoxides) or dithiol crosslinking (like arsenite or phenylarsine oxide), the effects of $Cu(OP)_2$ are not mediated by the S site. Indeed, the latter is *blocked* by 15–20 μM NEM [9,10], while the effects of $Cu(OP)_2$ are *potentiated* by these concentrations of NEM (Fig. 1). We must conclude that the effects of $Cu(OP)_2$ depend on oxidation of a previously unrecognized thiol site which is not accessible to other oxidants used to induce pore opening. It is tempting to speculate that this site coincides with the cryptic site(s) unmasked by substitution with NEM. In this scenario, $Cu(OP)_2$ would be the only oxidant able to interact with this novel site in the absence of reaction of the primary site with a substituting agent. Alternatively, it must be postulated that the $Cu(OP)_2$-reactive site is differ-

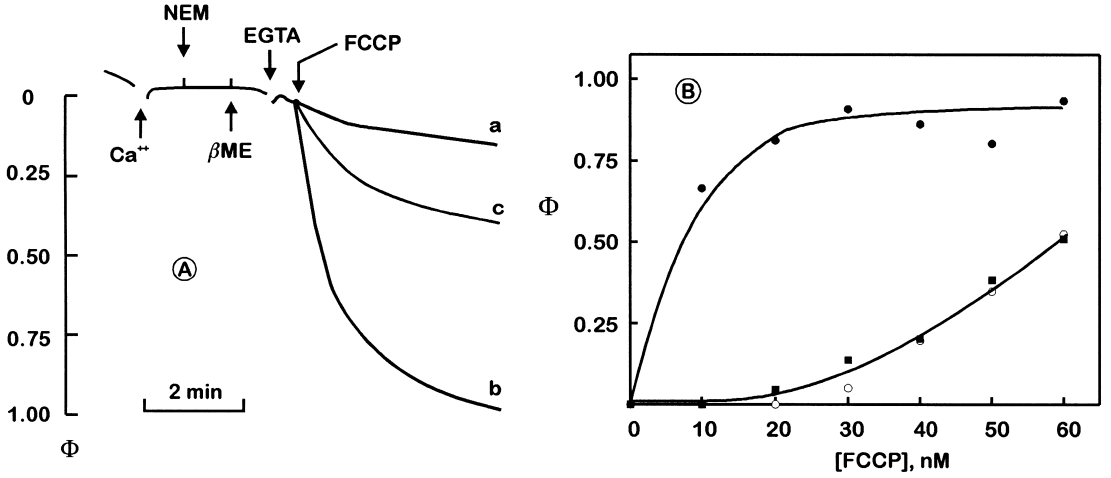

Fig. 4. Effect of β-ME on NEM potentiation of the fraction of mitochondria permeabilized by FCCP. Experimental conditions were as in Fig. 1. (A) Where indicated, 5 μM Ca^{2+}, 1 mM NEM (traces b and c), 1 mM β-ME (trace c), 0.5 mM EGTA and 30 nM FCCP were added. (B) The experiments were carried out exactly as shown in panel A. The indicated concentrations of FCCP were added after Ca^{2+} alone (open circles), or after treatment with 1 mM NEM (closed circles) or with 1 mM NEM plus 1 mM β-ME (closed squares). Φ denotes the fraction of mitochondria permeabilized to sucrose by FCCP.

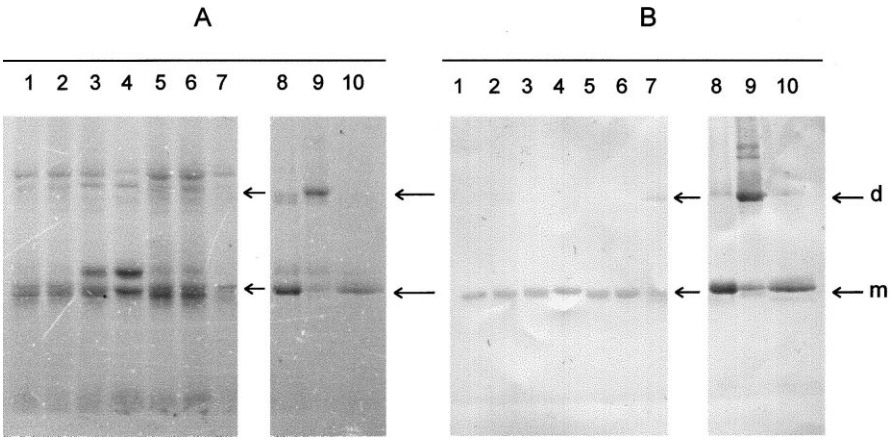

Fig. 5. SDS-PAGE and Western blot analysis of partially purified ANT after treatment with several reagents. Lack of ANT dimerization despite pore opening. Rat liver mitochondria (lanes 1–7) were incubated as described in detail in Section 2. After 1 min, mitochondria were either treated with 10 μM Ca^{2+} alone (a condition which was not accompanied by pore opening under these conditions), or with Ca^{2+} followed by reagents that all caused pore opening within 15 min ($Cu(OP)_2$ and NEM alone or in combination, arsenite, *tert*-butylhydroperoxide, or diamide). After 15 min, mitochondria were extracted with Triton X-100, the ANT was partially purified by HAP chromatography, and samples were analyzed by SDS-PAGE (12% acrylamide-0.3% bisacrylamide) followed by transfer onto nitrocellulose and Ponceau red staining (panel A), and by Western blotting with an ANT antibody after destaining of the same filter (panel B). Lanes 1, 10 μM Ca^{2+} alone; lanes 2, 2.5 μM $Cu(OP)_2$; lanes 3, 25 μM NEM and 2.5 μM $Cu(OP)_2$; lanes 4, 1 mM NEM; lanes 5, 1 mM arsenite; lanes 6, 10 μM *tert*-butylhydroperoxide; lanes 7, 0.1 mM diamide. Bovine heart SMPs (5 mg per incubation) were extracted exactly as described in Section 2 either without further treatments (lanes 8), or after addition of 0.1 mM $Cu(OP)_2$ (lanes 9) or of 2 mM NEM followed by 0.1 mM $Cu(OP)_2$ (lanes 10). After partial purification of the ANT by HAP chromatography, samples were analyzed exactly as described above for mitochondria. Arrows marked 'm' and 'd' denote the expected position of the ANT monomer and dimer, respectively, as determined from authentic standards included in each gel (omitted for clarity).

ent from the one unmasked by NEM, and that dithiol-disulfide interconversions at multiple sites contribute to modulation of the probability of pore opening.

Since $Cu(OP)_2$ only oxidizes surface SH groups in intact mitochondria [27], we suggest that the relevant $Cu(OP)_2$-reactive sites are either located on the outer surface of the inner membrane, or on intermembrane/outer membrane regulatory protein(s), possibly at contact sites. This is again at variance from the S site which, being oxidized by mitochondrial GSSG [12], must be exposed to the matrix side of the inner membrane.

4.3. PT induction by $Cu(OP)_2$ and NEM is not mediated by dimerization of the ANT

An obvious candidate for the membrane permeabilizing effects of $Cu(OP)_2$ (and, by inference, of NEM) is the ANT. Based on the effects of two inhibitors of the ANT (bongkrekate, which inhibits the PT, and atractylate, which promotes it), this protein has long been suspected to participate, directly or indirectly, in the PT ([28], see [7] for a critical assessment, and [29,30] for relevant experiments). Under well-defined conditions both $Cu(OP)_2$ and substituted maleimides react with ANT cysteines. In SMP, $Cu(OP)_2$ can induce the formation of disulfide bridges between Cys^{56} residues on adjacent ANT molecules, while NEM can react with three ANT cysteines [18]. Since $Cu(OP)_2$ only oxidizes surface residues, and since the reactive Cys^{56} are located within the matrix M1 loops which are not accessible to the oxidant in intact mitochondria [18,26,27], the present finding that the ANT does not dimerize after treatment of mitochondria with $Cu(OP)_2$ is consistent with current models of the ANT [18,26]. It should be emphasized that our results do not exclude that the ANT can take part in pore formation after modification by NEM and other SH reagents (see, e.g. [31–33]). However, based on the results described in this paper it can be ruled out that ANT dimerization is an essential feature of pore formation by $Cu(OP)_2$, NEM, and S site reagents like *tert*-butylhydroperoxide and diamide, at variance from a recent suggestion [34].

4.4. Conclusions

The present results confirm the key role of SH groups in regulation of the permeability transition pore, an issue which has long been appreciated in the field (e.g., [14–18,35] and [1,2] for reviews). The specific contribution of this work is the identification of a novel, cryptic site which is unmasked by thiol substitution with NEM, and which might coincide with the surface site reactive with $Cu(OP)_2$. This oxidizable site (i) does not coincide with the previously identified S site, which is in redox equilibrium with the matrix glutathione pool [9–12]; and (ii) is not located on the ANT. Although PT is an extremely complex phenomenon, it appears that dissection of its regulatory features with chemical modification (see also [36]) is providing critical information which should be useful in the design of strategies aimed at MTP identification and purification.

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