Modulation of the Mitochondrial Permeability Transition Pore by Pyridine Nucleotides and Dithiol Oxidation at Two Separate Sites*

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After accumulation of a Ca\(^{2+}\) load, the addition of uncoupler to respiring rat liver mitochondria is followed by opening of the permeability transition pore (MTP), a voltage-dependent channel sensitive to cyclosporin A. The channel’s voltage threshold is profoundly affected under conditions of oxidative stress, with a shift to more negative values that may cause MTP opening at physiological membrane potentials. In this paper we further clarify the mechanisms by which oxidative agents affect the apparent voltage dependence of the MTP. We show that two sites can be experimentally distinguished. (i) A first site is in apparent oxidation-reduction equilibrium with the pyridine nucleotide (PN) pool (NADH/NAD + NAPDH/NADP); PN oxidation is matched by increased MTP open probability under conditions where the glutathione pool is kept in the fully reduced state; this site can be blocked by N-ethylmaleimide but not by monobromobimane, a thiol-selective reagent. (ii) A second site coincides with the oxidation-reduction-sensitive dithiol we have recently identified (Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S., and Bernardi, P. (1994) J. Biol. Chem. 269, 16638-16642); dithiol cross-linking at this site by arsenite or phenylarsine oxide is matched by increased MTP open probability under conditions where the PN pool is kept in the fully reduced state; at variance from the first, this site can be blocked by both N-ethylmaleimide and monobromobimane and is probably in equilibrium with the glutathione pool. Based on these findings, we reassess the mechanisms by which many oxidative agents affect the MTP and resolve conflicting reports on the relative role of PN and glutathione oxidation in the permeability transition within the framework of MTP (dys)regulation at two separate sites.

Mitochondrial function is affected by oxidative stress, as shown by the permeability increase caused by a wide variety of oxidants (Ref. 1 and references therein). The permeability increase is favored by Ca\(^{2+}\) accumulation and causes equilibria of solutes up to ~1500 Da in molecular mass, depolarization with uncoupling, and release of the previously accumulated Ca\(^{2+}\) (see Refs. 2-4 for recent reviews). Most authors now agree that this phenomenon (the "permeability transition" of Hunter and Haworth (5-7)) is due to opening of the permeability transition pore (MTP).\(^1\) The MTP is an inner membrane channel (5-7) inhibited by CsA (8-11), which, by a number of criteria (12-15), appears to coincide with the mitochondrial megachannel discovered by patch clamp studies of rat liver mitoplasts (16-18).

The mechanism(s) by which oxidants induce opening of the MTP has been the subject of many studies, often carried out without realizing that the permeability transition was at least partially involved (see the thorough discussion by Zoratti and Szabo(14)). Work by Lehninger and co-workers pointed to the role of the oxidation-reduction level of PN in the modulation of mitochondrial Ca\(^{2+}\) fluxes, showing that PN oxidation caused Ca\(^{2+}\) efflux, which could be reversed by PN reduction (19).

Several subsequent studies, however, showed that Ca\(^{2+}\) efflux and the redox state of PN could be dissociated (20, 21) and that PN oxidation could be the consequence rather than the cause of the permeability transition and therefore of Ca\(^{2+}\) efflux (22). These observations undermined the PN hypothesis of regulation of Ca\(^{2+}\) efflux, now ascribed to MTP opening (4).

Another potential target for oxidant species that received considerable attention in MTP (dys)regulation is mitochondrial glutathione (Ref. 4 and references therein). In an influential study, Pfeiffer and co-workers (23) were able to independently modulate the NAD/NADH, NADP/NADPH, and GSSG/GSH ratios and concluded that the latter was the relevant factor since permeabilization could be observed after glutathione oxidation under conditions where the PN pool was kept fully reduced. This correlation was subsequently challenged by the finding that the permeability transition induced by organic hydroperoxides could be inhibited by butylhydroxytoluene while glutathione remained oxidized (24) and by the observation that oxidation of glutathione with 1,3-bis(2-chloroethyl)-1-nitrosourea (which prevents PN oxidation by inhibiting glutathione reductase) was not followed by Ca\(^{2+}\) efflux due to a permeability transition (25).

The effect of several oxidative agents on mitochondrial membrane permeability has recently been rationalized within the framework of the MTP voltage dependence (1, 26). The apparent pore gating potential (i.e. the threshold voltage for pore

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1 The abbreviations used are: MTP, mitochondrial permeability transition pore; NEM, N-ethylmaleimide; CsA, cyclosporin A; Δψ, transmembrane potential difference; FCCP, carbonylcyanide-p-trifluoromethoxyphenyl hydrazone; MBB, monobromobimane; AaC, acetacetate; BOH, β-hydroxybutyrate; DIA, diamide; AsO, AsO; PhAsO, phenylarsine oxide; TBH, tert-butyhydroperoxide; PN, pyridine nucleotide(s); Mops, 4-morpholinepropanesulfonic acid.
opening) is shifted to more negative $\Delta \psi$ levels by dithiol oxidation, resulting in a higher probability of pore opening at physiological membrane potentials (1). Conversely, dithiol reduction or substitution with NEM is accompanied by a shift of the apparent gating potential to less negative $\Delta \psi$ levels, resulting in a lower probability of pore opening (1). The dithiol responsible for these effects can be selectively blocked by derivatization with MBM, which can therefore be used as a probe for the pore agonists that act at this site (27).

Several open questions remain. Is the oxidation of the MBM- and NEM-sensitive dithiol linked to changes in the oxidation-reduction state of mitochondrial PN and/or glutathione? Is there a role for PN and glutathione oxidation in regulation of the apparent MTP voltage dependence, and what is their relative contribution? In this paper we describe the properties of two distinct sites that affect the probability of MTP opening through oxidation-reduction reactions. The first site is blocked by NEM but not by MBM and appears to be in redox equilibrium with the PN pool. The second site is blocked by MBM, coincides with the oxidation-reduction-sensitive dithiol we have recently identified (1), and is presumably in equilibrium with the glutathione pool. The existence of at least two oxidation-reduction-sensitive regulatory sites on the MTP in equilibrium with both PN and glutathione readily resolves many conflicting reports in the literature and allows a better description of the MTP response to oxidative stress under a variety of in vitro conditions.

**Materials and Methods**

Rat liver mitochondria were prepared as described (28). The fraction of mitochondria permeabilized to sucrose by MTP opening and the membrane potential were determined as described in detail elsewhere (Refs. 29 and 30, respectively).

PN and glutathione levels were determined after preincubation of mitochondria under the conditions specified in the table legends. The sequence of the addition of Ca$^{2+}$, EGTA, inducers, and inhibitors was as shown for Fig. 1, while rotenone, when present, was included from the beginning of the experiments. Incubation conditions were identical to those of the light-scattering experiments, except that the FCCP concentration was 200 nM. PN or glutathione determinations were carried out 1 min after the addition of FCCP. NADH and NADPH levels were determined in alkaline extracts and GSH level was determined on acid extracts based on coupled enzyme reactions exactly as described in Ref. 23. For the glutathione measurements, the concentration of mitochondria was increased up to 2 mg of protein $\times$ ml$^{-1}$ with a corresponding increase of all reagents (EGTA, Ca$^{2+}$, FCCP, CsA, AsO, AcAc, etc.).

Since the total thiol content determined with 5,5'-dithiobis(2-nitrobenzoic acid) at 412 nm in the same extracts gave exactly the same values of the direct glutathione measurements, the latter procedure was routinely used. All chemicals were of the highest purity commercially available. Incubation conditions and further experimental details are given in the figure legends.

**Results**

Differential Sensitivity to BOH and MBM of the Effects of AcAc and AsO. Respectively—In the experiments of Fig. 1, rat liver mitochondria energized with succinate in the presence of rotenone were incubated in a sucrose-based medium containing 1 mM $P_i$. The light scattering of the mitochondrial suspension at 540 nm was monitored, and the fraction of mitochondria permeabilized to sucrose as a result of MTP opening was determined as described (29). After the accumulation of a 20 nM Ca$^{2+}$ pulse, EGTA was added to prevent Ca$^{2+}$ redistribution at the moment of uncoupler addition, followed by 40 nM FCCP (causing a depolarization of about 40 mV from the initial value of about $-200$ mV, see e.g. Figs. 3 and 4). This concentration of uncoupler was not able to open the MTP, indicating that under these conditions the threshold voltage for MTP opening was less negative than about $-160$ mV (Fig 1, A and B, traces a).

When 0.5 mM AcAc was added after Ca$^{2+}$ accumulation, however, the addition of FCCP was followed by MTP opening in a large fraction of mitochondria (panel A, trace b). The effect of AcAc was completely prevented by NEM (panel A, trace d) but not by MBM (panel A, trace c). It is well known that the addition of AcAc in the presence of rotenone oxidizes mitochondrial PN and that this effect is counteracted by BOH (see Table I and Refs. 4 and 31 for its relation to MTP opening). Interestingly, the shift caused by AcAc on the threshold voltage for MTP opening was counteracted by BOH (panel A, trace e). It should be mentioned here that, at variance from BOH (see Table I), NEM did not prevent PN oxidation by AcAc (not shown).

In the experiments of Fig. 1B, the shift of MTP response to depolarization was induced with AsO, a selective dithiol crosslinker (32) and well characterized MTP agonist (1, 27). The addition of 40 nM FCCP was again followed by MTP opening, which could be counteracted by both NEM (panel B, trace d; cf. Ref. 1) and MBM (panel B, trace c; cf. Ref. 27). The novel finding of this experiment is that the effect of AsO (or of its analog PhAsO, not shown) could not be blocked by BOH (panel B, trace e), which instead blocked the effects of AcAc (compare panels A and B).

**Table I**

| Additions | NADH | NADPH | GSH |
|-----------|------|-------|-----|
| Rotenone  | 100  | 100   | 100 |
| Rotenone, AcAc, FCCP | 29.6 ± 0.6 | 41.3 ± 5.5 | 102.6 ± 15.1 |
| Rotenone, AsO, FCCP | 118.3 ± 29.4 | 102.6 ± 16.6 | 106 ± 14.8 |
| FCCP      | <10  | <10   | 104.8 ± 27.2 |
| Rotenone, FCCP | 82.3 ± 8.7 | 96 ± 2.6 | 121.3 ± 2.7 |
Relationship between Reduced PN and Glutathione Levels and MTP Gating Profile—Mitochondrial PN are in oxidation-reduction equilibrium with matrix glutathione through PN transhydrogenase and glutathione reductase (see e.g. Refs. 23 and 34). Thus, depending on the experimental conditions glutathione oxidation may or may not cause PN oxidation and vice versa, which makes correlations between MTP open probability and oxidation of PN and/or glutathione difficult to establish a priori. To address the relative role of PN and glutathione oxidation in the shifts of MTP voltage dependence observed here we have carried out parallel determinations of NADH, NADPH, and reduced glutathione levels.

The MTP response to graded additions of FCCP is somewhat variable in different mitochondrial preparations (see Refs. 1 and 29 for a discussion). In these experiments we have therefore chosen FCCP concentrations giving maximal uncoupling. The following results give therefore an upper limit for PN or glutathione oxidation under the experimental conditions of Figs. 1–4. Table I shows that the addition of FCCP in the presence of rotenone did not appreciably oxidize PN or glutathione. As expected, NADH and NADPH oxidation could be achieved by either the addition of AcAc, or the omission of rotenone, both conditions affecting the MTP response to depolarization (see Figs. 3 and 4). On the other hand, under both conditions glutathione remained fully reduced, and the difference between reduction levels of PN and glutathione was particularly large in the case of rotenone omission. These data indicate that under our experimental conditions the PN oxidation-reduction state is related to the MTP gating profile independently of changes in the oxidation-reduction state of glutathione.

Table I also shows that in the presence of rotenone and FCCP the addition of AsO, which under these conditions causes pore opening, was not accompanied by oxidation of either PN or glutathione. The experiment therefore indicates that the oxidation-reduction-sensitive dithiol (1) can affect the MTP gating profile independently of changes in the oxidation-reduction state of PN. It appears likely that the AsO-reactive dithiol is in equilibrium with glutathione and that the AsO-complexed species, which is equivalent to the disulfide for MTP regulation (1), cannot be reduced by glutathione and becomes thus unavailable for its natural oxidation-reduction partner (see also Table III).

In the experiments of Table I CsA was included to prevent any secondary effects of MTP opening on reduced PN and glutathione levels. In the experiments of Table II, CsA was omitted and MTP opening was induced by AsO plus FCCP (i.e. by a condition that does not affect the level of reduced PN when the MTP is blocked by CsA; Table I). Under these conditions...
PN oxidation could be reproducibly observed, confirming earlier reports that PN oxidation may well follow rather than precede the permeability transition (22, 23, 34). To rule out the possibility that CsA was preventing PN oxidation by mechanism(s) unrelated to its inhibitory effects on the pore, we have measured PN and glutathione levels in AsO- and FCCP-treated mitochondria in the absence of CsA, but under conditions where MTP opening was prevented by the addition of MBM, NEM, or EGTA. The results, also presented in Table II, indicate that MBM prevented reduced irrespective of the method used to prevent pore opening. These data indicate that PN oxidation is more likely to be the consequence than the cause of MTP opening when dithiol cross-linkers like AsO or PhAsO are used as the agonists, at variance from a recent suggestion to the contrary (35).

TBH and DIA Oxidize both PN and Glutathione under Conditions That Promote MTP Opening by FCCP—In the experiments of Table III we tested the effects of the organic hydroperoxide, TBH and of the dithiol oxidant, DIA, on the levels of reduced PN and glutathione. These widely used pore agonists (eg. Ref. 1) were both able to promote oxidation of PN and glutathione, as reported by many authors (eg. Ref. 23), and their effect was more pronounced on the levels of NADH. The addition of NEM and MBM, which block the effects of TBH and DIA on the pore (1, 27) partially prevented oxidation of NADH induced by TBH and DIA, while NEM but not MBM prevented oxidation of NADPH. Since both NEM and MBM form adducts with reduced glutathione in the matrix (no reduced glutathione could be detected in their presence; Table III), these data are in full agreement with earlier observations that oxidation of mitochondrial PN by TBH is only possible through the combined action of glutathione peroxidase, glutathione reductase, and PN transhydrogenase, and it is therefore prevented when glutathione is not available (36). Furthermore, these data suggest that DIA may be affecting the MTP through the same pathway as TBH.

**DISCUSSION**

In this paper we have shown that the apparent MTP gating potential can be modulated by oxidation-reduction effectors at two sites that can be distinguished experimentally. One site (which we will now call the “P site”) appears to be modulated through the oxidation-reduction state of PN even when glutathione is fully reduced, and it accounts for the effects of MTP agonists like AcAc (19) or duroquinone (37). The P site can be blocked by NEM but not by MBM. The other site (which we will now call the “S site”) coincides with the oxidation-reduction-

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**TABLE II**

| Additions          | Metabolites | NADH | NADPH | GSH | % |
|--------------------|-------------|------|-------|-----|---|
| None               | 100         | 100  | 100   | 66.2| 6 |
| AsO, FCCP          | 47.5 ± 3.7  | 66.2 ± 12.2 | 103.8 ± 9.8 | 6 |
| AsO, FCCP, MBM     | 97.6 ± 10   | 101 ± 6.7 | NM   | 6 |
| AsO, FCCP, NEM     | 86.3 ± 3.8  | 87.3 ± 9.6 | NM   | 6 |
| AsO, FCCP, EGTA    | 100.7 ± 8.6 | 91.7 ± 7.5 | 106.3 ± 10.3 | 6 |

**TABLE III**

| Additions          | Metabolites | NADH | NADPH | GSH | % |
|--------------------|-------------|------|-------|-----|---|
| None               | 100         | 100  | 100   | 66.2| 6 |
| TBH                | 22.8 ± 14.5 | 63.9 ± 8.2 | 26.4 ± 3.4 | 6 |
| TBH, MBM           | 62.8 ± 29.2 | 63.9 ± 32.3 | NM   | 6 |
| TBH, NEM           | 85.2 ± 91.1 | 92.4 ± 12.6 | NM   | 6 |
| DIA                | 29.5 ± 2.2  | 78.7 ± 10.3 | 15 ± 7.3 | 6 |
| DIA, MBM           | 46.3 ± 8.5  | 50.4 ± 3.8 | NM   | 6 |
| DIA, NEM           | 66.2 ± 2.8  | 83.5 ± 8.5 | NM   | 6 |

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Note that the standard error is extremely large in the measurements with TBH, particularly in the presence of MBM. We have no explanation for this variability, which is anomalous relative to all the other conditions tested.
sensitive dithiol (1), and it can be activated by reaction with AsO or PhAsO even when the PN pool is fully reduced. The S site can be blocked by both NEM and MBM. It appears that oxidants like TBH and DIA can oxidize both PN and glutathione and thus affect both the P and S sites. Irrespective of the precise mechanism by which glutathione and PN affect the MTP, oxidative stress causes an increased probability of pore opening when the concentrations of reduced glutathione and PN decrease.

Regulation of the MTP by Both PN and Glutathione—The relative role of PN and glutathione in controlling mitochondrial membrane permeabilization to solutes has been addressed in a large number of studies, but the results are contradictory, and an unequivocal answer is still missing (see Ref. 4 for a critical analysis and references therein for all the original contributions). In an early study, Pfef?er and co-workers (23) addressed this issue by correlating pore opening by TBH plus Ca2+ to the oxidation-reduction state of mitochondrial PN and glutathione. Ca2+-dependent permeabilization was observed (i) when NADH, NADPH, and glutathione were oxidized; (ii) when NADH and glutathione, but not NADPH, were oxidized; and (iii) when PN were fully reduced and only glutathione was oxidized. It was inferred that only the oxidation of glutathione was relevant to membrane permeabilization and that PN oxidation was more likely to follow than to precede pore opening (23). The only missing condition in (23) was one where PN were fully oxidized while the glutathione pool was kept in the reduced state. Such a condition is described in the present paper and has revealed an independent role for PN in MTP regulation, as suggested in early work on the permeability transition (reviewed in Ref. 4).

Our findings demonstrate that MTP opening is correlated to both the oxidation-reduction state of PN through the P site and (presumably) of glutathione through the S site. We would like to stress that there is no direct evidence here or in the literature that glutathione as such is involved in pore regulation. Indeed, many glutathione oxidants could also directly oxidize the dithiol S site at the same time. Furthermore, since PN and glutathione are in oxidation-reduction equilibrium through PN transhydrogenase, glutathione reductase, and possibly mitochondrial thioredoxin reductase (38), reagents like TBH and DIA can lead to oxidation of both glutathione and PN, thus affecting both sites at the same time.

While the NEM- and MBM-sensitive S site coincides with the dithiol we recently identified (1, 27), the NEM-sensitive P site remains chemically undefined. Indeed, although the NEM reactivity may suggest that a cysteine thiol group is involved, no other compound we tested among a variety of thiol reagents (including mersalyl and methyl methanethiosulfonate, data not shown) was able to block the P site.

In principle, NAD(P)H (we could not resolve which PN is responsible, even if the data of Table III would suggest a better correlation with NADH levels) might affect the MTP as a reductant, possibly through a voltage-sensing element that could also be reduced by glutathione. However, it should be mentioned that the P site is insensitive to reductants like dithiothreitol and β-mercaptoethanol (data not shown). An alternative possibility is that PN may directly interact with the P site modulating the MTP response to voltage by an allosteric mechanism, which is consistent with the NADH-specific inhibitory effect on the MTP reported by Hunter and Haworth (5, 39) in deenergized mitochondria. An example of regulation of a channel by PN can be found for the voltage-dependent anion channel of the outer mitochondrial membrane, where NADH (but not NADH, NADPH, or NADP+) decreases the permeability to ADP by a factor of 6 (40).

The concept that the MTP is modulated by oxidizing agents at two sites resolves several standing problems. It is clear from the experiments presented here that the PN and glutathione oxidation-reduction state does not necessarily affect MTP opening in an all-or-nothing fashion. In other words, pore opening may or may not follow PN and/or glutathione oxidation, depending on additional factors like e.g. the magnitude of the membrane potential, the Ca2+ load, and the level of endogenous adenine nucleotides. Yet, under oxidative conditions the apparent gating potential is demonstrably shifted toward the resting level (Figs. 3 and 4). This explains why MTP opening (and the ensuing Ca2+ efflux) can be dissociated from the oxidation-reduction state of PN and glutathione and why an unequivocal correlation between MTP opening and oxidation-reduction levels of either glutathione or PN alone could not be established, even if it is widely accepted in the field that pore operation is indeed affected by the mitochondrial redox state (4).

Analogies with Regulation of Voltage-gated K+ Channels—We have already pointed out the similarities between many aspects of MTP modulation and those of well-characterized channels like the N-methyl-o-aspartate and the ryanodine receptors (3, 41). Interesting analogies also exist with voltage-gated K+ channels (Kv channels).

The voltage inactivation properties of the αβ Kv channels can be modulated by glutathione through a cysteinyl residue close to the N terminus. This residue can be located on either the pore-forming α subunit in rapidly inactivating (A-type) Kv channels (42) or on the regulatory β1 subunit, which confers the rapidly inactivating mode to otherwise slowly inactivating Kv channels (43). In either case, rapid inactivation may involve formation of a disulfide with a second cysteinyl residue in an as yet unidentified channel region (42, 43). Also relevant to the present discussion, it has been noted that Kv channel β subunits bear a striking resemblance (both in terms of primary sequence and of secondary structural elements) to the superfamily of NAD(P)H-dependent oxidoreductases, with conservation of the PN binding site, suggesting that PN binding could be involved in Kv channel regulation (44). It must be stressed that oxidation-reduction-sensitive K+ channels exist in Escherichia coli (45), which bear sequence homology to the mammalian and Drosophila αβ Kv channels (46). The MTP might have conserved structural features of the archetypal K+ channel and thus accomplish modulation of its voltage-dependence by glutathione and PN by similar mechanisms.

Conclusions and Perspectives—We conclude that (i) the MTP is modulated by the oxidation-reduction state of mitochondria through both PN and (presumably) glutathione at the P and S sites, respectively, and (ii) suggest that this modulation is accomplished through a change of the threshold potential for MTP opening. Mitochondrial (dys)function during oxidative stress is now increasingly considered as a key event in a variety of forms of cell death, ranging from ischemia (47–51) to excitotoxic neurodegeneration (52) to oxidant-induced stress (53) to apoptosis (54, 55). Because of its exquisite sensitivity to oxidative stress the MTP appears as a likely target on which many pathological agents or conditions may converge. Definition of the structural features that make the MTP sensitive to oxidation-reduction events at the P and S sites may well lead to novel tools for therapeutic intervention, and it is a major challenge for future research in our laboratory.

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