Selective inhibition of the mitochondrial permeability transition pore at the oxidation–reduction sensitive dithiol by monobromobimane

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Abstract In this paper we introduce monobromobimane, a thiol reagent, as a selective blocker of the recently identified dithiol whose oxidation–reduction status modifies voltage sensing by the mitochondrial permeability transition pore, a cyclosporin A-sensitive channel. Monobromobimane does not inhibit the phosphate carrier, nor does it interfere with Ca^{2+} transport, energy coupling or ATP production and transport. We show that monobromobimane selectively prevents the shift in pore gating potential caused by some dithiol oxidants or crosslinkers but not by increasing $[Ca^{2+}]$, allowing a clear distinction of the pore agonists which act at this site.

Key words: Mitochondrial channel; Cyclosporin A; Membrane permeability (rat liver)

1. Introduction

The mitochondrial permeability transition pore (MTP) is an inner membrane channel activated by matrix Ca^{2+}, inhibited by matrix H^+ and by cyclosporin A, and modulated by an array of effectors acting at discrete sites [1–6] (see also [7,8] for reviews). We have recently identified the membrane potential as a key MTP effector, showing that the pore behaves as a voltage-dependent channel exhibiting an increased open probability upon depolarization [9]. In a recent study investigating the mechanism of voltage sensing by the MTP, we have described a critical dithiol whose oxidation–reduction state modulates the pore response to a given membrane potential. Dithiol oxidation or cross-linking is associated to a higher open probability, and this effect can be reversed by disulfide reduction or prevented by thiol substitution with *N*-ethylmaleimide (NEM) [10]. Here we show that monobromobimane (MBM), a *syn*-9,10-dioxabimane bromoderivative which selectively reacts with thiol groups [11], prevents the shift in MTP gating potential caused by some dithiol oxidants or crosslinkers. At variance from NEM, MBM does not inhibit the P_i carrier, nor does it interfere with Ca^{2+} transport, energy coupling or ATP production and transport. MBM is thus a selective effector of the MTP at the above-mentioned dithiol, allowing a discrimination of the pore agonists which act at this site. Since upon binding to protein thiol groups a fluorescent product is formed, MBM can also become an important tool for sensor tagging and isolation. A portion of this work has been presented in Abstract form [12].

2. Materials and methods

Rat liver mitochondria were prepared as described previously [13], except that the last washing included 0.1 mM EGTA-Tris while bovine serum albumin was omitted. The fraction of mitochondria permeabilized to sucrose, a measure of MTP opening, and mitochondrial membrane potential were determined exactly as described in [14] and [15], respectively. Respiration was determined with a Clark oxygen electrode (Yellow Springs Instruments, Ohio), and the rate of P_i transport was determined as described in [16]. Ca^{2+} fluxes were followed at 650 minus 690 nm with an Aminco DW2a dual wavelength spectrophotometer in incubations containing 25 μM Arsenazo III and 20 μM Ca^{2+}. The rate of Ca^{2+} uptake on the Ca^{2+} channel (uniporter) was measured with the method of Bragadin et al. [17] in respiratory-inhibited mitochondria incubated in a sucrose-based medium. Ca^{2+} uptake was initiated by the addition of excess valinomycin, thus ensuring that the flux was measured under true rate-limiting conditions [17]. The rate of Ca^{2+} efflux on the Na^+-independent pathway was studied by adding 0.1 mM ruthenium red to succinate-energized mitochondria which had accumulated 40 nmol $Ca^{2+} \cdot$ mg protein^{-1} in the presence of 1 mg $\cdot$ ml^{-1} cyclosporin A. MBM and trimethylammonium-MBM (THIOLYTE) were purchased from Calbiochem, and all chemicals were of the highest purity commercially available. Incubation conditions and details of the experimental protocols are given in the figure legends.

3. Results

The MTP voltage dependence can be studied in a mitochondrial population by following the fraction of mitochondria which become permeable to sucrose (an indication of MTP opening) as a function of the membrane potential ($\Delta\gamma$), as varied with different concentrations of the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) [14]. Conditions have to be chosen so as to prevent matrix acidification (which would inhibit the MTP), e.g. by P_i, and Ca^{2+} efflux, e.g. by Ruthenium red or EGTA [14]. The lower the concentrations of FCCP needed to open the pore, the higher the gating potential, which can be measured from $\Delta\gamma$ determinations of parallel samples treated with cyclosporin A [14].

The experiments of Fig. 1 show that after accumulation of a small Ca^{2+} load in the presence of 1 mM P_i the addition of 30 nM FCCP, corresponding to a depolarization of about 45 mV, was not sufficient to trigger MTP opening (panel 1, trace a). If the addition of FCCP was preceded by that of the arsenite anion (AsO), on the other hand, identical concentrations of

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Abbreviations: MTP, mitochondrial permeability transition pore; $\Delta\gamma$, transmembrane potential difference; EGTA, ethylene-bis(oxoethylenenitrilo) tetraacetic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; NEM, *N*-ethylmaleimide; MBM and MBM$^+$, monobromobimane and trimethylammonium monobromobimane, respectively; DIA, diamide; AsO, arsenite anion.

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FCCP caused now pore opening in >75% of the mitochondria, indicating a shift of gating potential to higher values (panel 1, trace b). This shift could be prevented by increasing concentrations of MBM (panel 1, traces c–f), as is also the case for the sulfhydryl group reagent, NEM [10]. Similar effects of MBM were observed if the shift of the responding population was obtained with the addition of the dithiol oxidant, diamide (DIA) rather than of AsO (see Fig. 3). Fig. 1, panel 2, shows the concentration-dependence of the MBM inhibition, which displayed an apparent I_{50} of about 7 μM (closed symbols). At variance from MBM, a positively charged trimethylammonium MBM derivative (MBM^+) was completely ineffective (open symbols) even at a concentration of 100 mM (data not shown). Parallel titrations of the membrane potential as a function of the FCCP concentration in the absence or presence of AsO and of MBM, and including cyclosporin A to block pore opening, indicated identical dose–response curves (not shown, but see Fig. 1 [10]). Thus, the effects of MBM are not due to a direct modification of the $\Delta\gamma$, nor to a modification of its response to uncoupler. Rather, MBM appears to prevent the shift in gating potential caused by dithiol cross-linking or oxidation, most likely by reacting with the same NEM-sensitive site characterized previously [10].

In the experiments of Fig. 2 we measured the rate of P_i transport at increasing concentrations of MBM (closed symbols) or of NEM (open symbols). It can be clearly appreciated that, at variance from NEM (used here as a positive control [16]), MBM was totally ineffective as an inhibitor of the P_i carrier at concentrations which fully prevented the AsO-induced effects (compare with Fig. 1). Thus, MBM can be used

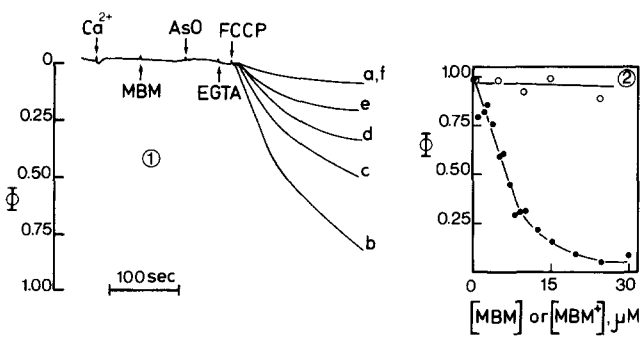

Fig. 1. Inhibitory effect of MBM, but not of a trimethylammonium MBM derivative, on MTP opening induced by a small depolarization in the presence of AsO. The incubation medium contained 0.2 M Sucrose, 10 mM Tris-MOPS pH 7.4, 5 mM Succinate-Tris, 1 mM P_i-Tris, 10 μM EGTA-Tris, 2 μM Rotenone, 0.5 $\mu g \cdot ml^{-1}$ oligomycin. Final volume 2 ml, 25°C. The experiments were started by the addition of 1 mg of mitochondria (not shown). Panel 1. Where indicated (the first addition being made exactly 1 min after mitochondria) the following additions were made: 10 μM Ca^{2+}, MBM (traces c–f only) 0.5 μM AsO (traces b–f), 0.5 mM EGTA and 30 nM FCCP. The final MBM concentration was 5 μM (trace c), 8 μM (trace d), 15 μM (trace e) or 25 μM (trace f). Note that the trace obtained after addition of 30 nM FCCP in the absence of MBM and AsO (trace a) was indistinguishable from that obtained in the presence of 0.5 μM AsO and 25 μM MBM (trace f). The traces were obtained by following the light scatter of the mitochondrial suspension at 540 nm, and were calibrated to obtain the fraction of permeabilized mitochondria (Φ) as described [14]. Panel 2. Dose-dependence of the effects of MBM (closed symbols) or of MBM^+ (open symbols) on the fraction of mitochondria with an open pore in protocols identical to those depicted in panel 1, where only selected traces had been reported for the sake of clarity.

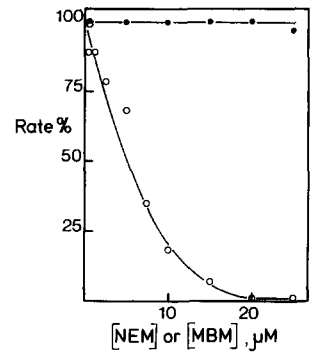

Fig. 2. Inhibition of P_i transport by NEM but not by MBM. NEM (open circles) or MBM (closed circles) were added to 1 mg of mitochondria in 0.1 ml of 0.25 M Sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EGTA-Tris in a stirred cuvette thermostatted at 25°C. After exactly one minute, 1.9 ml of 0.1 M NH_4-P_i, pH 7.4, 0.5 mM EGTA-Tris, 2 μM Rotenone were added. The rate of P_i transport was followed as the rate of absorbance change at 540 nm, and values on the ordinate are expressed as the percent of the rate observed in the absence of NEM or MBM. Values on the ordinate refer to the final concentrations of NEM or MBM during the assay.

to study the regulation of MTP voltage sensing under conditions where P_i fluxes are unaffected.

A set of experiments was carried out to determine whether MBM interferes with other mitochondrial functions which are relevant to operation of the MTP. We found (results not shown) that treatment of mitochondria with 50 nmol·mg protein^{-1} of MBM did not modify (i) the rate of basal or of uncoupler-stimulated respiration with succinate or glutamate/malate as substrates, indicating that MBM is neither an uncoupler nor a respiratory inhibitor; (ii) the ADP/O ratio, indicating that MBM does not interfere with either the adenine nucleotide translocator or the F_1F_0 ATPase; (iii) the rate of Ca^{2+} uptake on the Ca^{2+} channel (uniporter) measured under conditions where the transport reaction is rate limiting [17]; and (iv) the rate of Na^+-insensitive Ca^{2+} efflux after addition of ruthenium red to energized mitochondria that had accumulated 40 nmol Ca^{2+}·mg protein^{-1} in the presence of 1 $\mu g \cdot ml^{-1}$ cyclosporin A; the last two points indicate that MBM is not interfering with Ca^{2+} homeostasis. We must stress that in all cases the recordings obtained in the presence of MBM were indistinguishable from those obtained in its absence. As MBM does not influence Ca^{2+} fluxes and energy coupling we could then test its effects on the gating profile as modified by a variety of agents, including increasing Ca^{2+} concentrations.

Fig. 3 summarizes the results of a series of experiments where the FCCP-dependence of MTP opening in increasing fractions of mitochondria (Φ) was modulated by the addition of AsO or by an increase of the Ca^{2+} load. A different mitochondrial preparation was used for each set of experiments. Since the FCCP-dependence at a given Ca^{2+} load can vary depending on the concentration of intramitochondrial pore effectors (such as endogenous P_i, Ca^{2+}, Mg^{2+} and adenine nucleotides) and possibly on other unknown factors, we have obtained a similar 'basal' profile for each set of experiments by adjusting the Ca^{2+} concentration by trial and error (10 or 15 μM for panels 1 and 2, respectively) in order to obtain comparable profiles with both preparations (open circles in both panels). Then, the shift of MTP opening profile was obtained by either the addition of

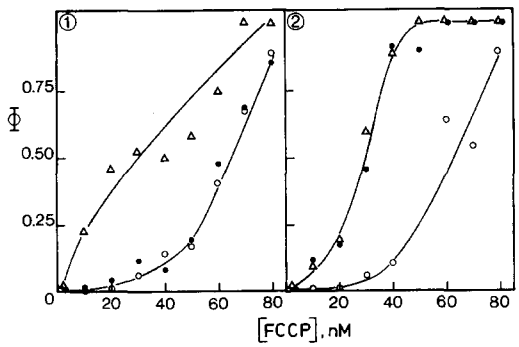

Fig. 3. Effect of MBM on the fraction of mitochondria permeabilized to sucrose at increasing FCCP concentrations. Incubation conditions and sequence of additions were as in Fig. 1. One minute after mitochondria, the following additions were made. Panel 1: 10 μM Ca^{2+}, 0.5 mM EGTA-Tris and FCCP (open circles); the same with 25 μM DIA added after Ca^{2+} (open triangles); the same with 25 μM MBM added before DIA (closed circles). Panel 2: 15 μM Ca^{2+}, 0.5 mM EGTA-Tris and FCCP (open circles); 30 μM Ca^{2+}, 0.5 mM EGTA-Tris and FCCP (open triangles); the same with 25 μM MBM added after 30 μM Ca^{2+} (closed circles).

DIA (panel 1) or by an increase of the Ca^{2+} load (panel 2) (open triangles in both panels). The experiments clearly document that MBM fully prevented the shift due to DIA (panel 1) but not that due to an increased Ca^{2+} load (panel 2) (closed circles).

4. Discussion

In this paper we have shown (i) that MBM, a selective thiol reagent [11], is able to specifically prevent the effects of DIA, a dithiol oxidant [18], and of AsO, a dithiol crosslinker [19], at a site which appears to modulate voltage sensing by the MTP [10]; (ii) that MBM does not affect the transport of P_i, Ca^{2+}, and adenine nucleotides; (iii) that respiration and ATP synthesis are not affected by this reagent. Thus, MBM behaves as a site-specific pore reagent lacking side effects on mitochondrial function.

In a previous paper we have shown that NEM has identical effects on the shift of MTP voltage sensing induced by DIA and AsO to those reported here for MBM [10]. Two problems, however, seriously limit the potential use of NEM in this capacity. The first problem is that NEM inhibits the P_i carrier [16] in the same concentration range as it modifies the putative voltage sensor [14]. P_i is an important factor regulating the MTP, and its effects are synergistic with those of Ca^{2+}, in part because of its buffering effects on matrix pH (see e.g. [20]). In a previous paper demonstrating the inhibitory effects of NEM on MTP modulation by the redox status of the DIA- and AsO-reactive dithiol the protocols had to be carefully defined to circumvent the potential problems posed by inhibition of the P_i carrier [10]. It is reassuring that identical results could be obtained with MBM (Figs. 1 and 2), and that the findings of the present work are fully consistent with our recent demonstration that mersalyl does not interact with the MBM- and NEM-reactive dithiol at concentrations which fully inhibit P_i transport [21]. The second problem is that NEM behaves as a pore inducer when used at concentrations which are only slightly higher than those needed to fully block the AsO- and DIA-sensitive site [14,22] thus limiting the useful NEM concentrations to a rather narrow range. None of these limitations applies to MBM as shown by its lack of effects on P_i transport (Fig. 2) and by its lack of activity as a pore inducer at concentrations up to 1 mM (data not shown). Thus, MBM is superior to NEM both in terms of specificity and of lack of side effects.

There is now little doubt that the MTP behaves as a voltage-dependent channel which increases its open probability upon depolarization ([8] and references therein). We proposed (i) that many signalling molecules and pharmacological agents could modulate the probability of pore opening by modifying its response to a given voltage (i.e., its voltage sensing) rather than by acting on the membrane potential itself [14]; and (ii) that an AsO- and DIA-sensitive dithiol was a factor in voltage sensing [10]. As shown here, the increased probability of pore opening at a given membrane potential observed at increasing Ca^{2+} loads ([14] and Fig. 3) is not mediated by the AsO and DIA-sensitive dithiol, since the Ca^{2+}-dependent shift of the FCCP dose–response curve for MTP opening was completely insensitive to MBM (Fig. 3).

It should be appreciated that MBM is not a pore inhibitor in an absolute sense, since it only prevents the modulatory effects of DIA and AsO rather than preventing MTP opening generally, and that its unique properties would have gone undetected in the traditional 'spontaneous' swelling assays. In the latter protocols, the MTP agonist or antagonist is added to a mitochondrial suspension at a fixed Ca^{2+} load, and the effect is then generally observed as a change in the apparent rate of spreading of the permeability transition through the mitochondrial population, which usually follows a lag phase of variable duration. The kinetics are always non-linear, and quantification is difficult and often deceptive because subpopulations of permeable and non-permeable mitochondria coexist, and Ca^{2+} is therefore continuously transferred from permeabilized to still impermeable mitochondria. This also causes secondary changes of membrane potential and of matrix pH (i.e. the other two main factors controlling the probability of MTP opening) in mitochondrial subpopulations, making quantification and establishment of correlations extremely difficult.

The protocol we introduced, namely the determination of the apparent voltage dependence of pore opening in increasing fractions of mitochondria [14] circumvents most of these problems because (i) the correlation between fraction of permeabilized mitochondria and absorbance or light scattering change is linear; (ii) the transition can be triggered simultaneously for all the responding mitochondria by depolarization after blockade of the Ca^{2+} channel with either ruthenium red or EGTA, thus preventing Ca^{2+} redistribution and all the secondary changes mentioned above; and (iii) the critical factors modulating MTP activity (matrix Ca^{2+} and pH, and transmembrane voltage) can be kept relatively constant and measured for the total population before the permeability transition is initiated [14]. Because of these features, this protocol allows an objective and reproducible quantification of the phenomenon under study. The only additional requirement is to perform independent titrations of the membrane potential to insure that the observed shifts do not depend on direct uncoupling or on respiratory inhibition [14]. It is clear that a shift in the *apparent* voltage dependence does not necessarily mean that the agonist or antagonist is acting through the putative MTP voltage sensor. Yet, as shown here, this method can allow a clear discrimina-

tion of whether different agonists act at the same or at different site(s), and recently allowed the identification of protoporphyrin IX as a powerful MTP agonist [23].

MBM^+, a derivative which has the same chemical reactivity of MBM but carries a net positive charge [11], is ineffective at blocking the MBM-sensitive site (Fig. 1). At variance from MBM, the former species does not readily permeate through cellular membranes [11], suggesting that the reactive site may be either internal or buried within the mitochondrial membrane(s). An alternative possibility is that the reactive site carries a net positive charge which would oppose the binding of MBM^+. The unequivocal assignement of pore effectors to definite channel domains or regulatory mechanisms must await pore isolation and reconstitution. Since upon its binding to protein thiol groups a fluorescent product is formed [11], MBM could also be useful for pore tagging and isolation. The feasibility of this approach is being actively tested in our laboratory.

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