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Switch from inhibition to activation of the mitochondrial permeability transition during hematoporphyrin-mediated photooxidative stress. Unmasking pore-regulating external thiols

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ABSTRACT

We have studied the mitochondrial permeability transition pore (PTP) under oxidizing conditions with mitochondria-bound hematoporphyrin, which generates reactive oxygen species (mainly singlet oxygen, $^1O_2$) upon UV/visible light-irradiation and promotes the photooxidative modification of vicinal targets. We have characterized the PTP-modulating properties of two major critical sites endowed with different degrees of photosensitivity: (i) the most photovulnerable site comprises critical histidines, whose photomodification by vicinal hematoporphyrin causes a drop in reactivity of matrix-exposed (internal), PTP-regulating cysteines thus stabilizing the pore in a closed conformation; (ii) the most photoresistant site coincides with the binding domains of (external) cysteines sensitive to membrane-impermeant reagents, which are easily unmasked when oxidation of internal cysteines is prevented. Photooxidation of external cysteines promoted by vicinal hematoporphyrin reactivates the PTP after the block caused by histidine photodegradation. Thus, hematoporphyrin-mediated photooxidative stress can either inhibit or activate the mitochondrial permeability transition depending on the site of hematoporphyrin localization and on the nature of the substrate; and selective photomodification of different hematoporphyrin-containing pore domains can be achieved by fine regulation of the sensitizer/light doses. These findings shed new light on PTP modulation by oxidative stress.

1. Introduction

The mitochondrial permeability transition (PT) features an abrupt increase of mitochondrial inner membrane permeability to solutes with molecular masses up to about 1500 Da. If long-lasting, this event is followed by membrane depolarization, matrix swelling, depletion of matrix pyridine nucleotides (PN), outer membrane rupture and release of intermembrane proteins, including cytochrome *c*, thus leading to cell death [1,2]. The PT is due to the opening of a protein channel, the CsA-sensitive permeability transition pore (PTP), whose structure has yet to be identified. The most studied candidates have been the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), and cyclophilin D (CyP-D) [1–4]. However, recent studies on the response of mouse mitochondria devoid of CyP-D [5–7], of ANT1 and ANT2 [8], and of the three VDAC isoforms [9] have shown that a functional PTP can still be formed in the absence of each of these proteins (see ref. [10] for a review). In spite of these uncertainties on the molecular nature of the PTP, its role as a mechanism for the execution of cell death is no longer questioned (reviewed in refs. [4] and [11]).

The PT can be favoured by a variety of structurally unrelated compounds and conditions. In spite of the inducer heterogeneity, a large body of data indicates that the PT is a regulated process, and that inducers converge on one or more discrete regulatory step(s) [12]. A key feature of the system is the regulation by both ΔpH and $\Delta\Psi_m$ components of the mitochondrial protonmotive force. Critical

Abbreviations: ANT, adenine nucleotide translocase; CsA, cyclosporin A; Cyp-D, cyclophilin D; $Cu(OP)_2$, copper-*o*-phenanthroline; Cys, cysteine; DTT, dithiothreitol; EGTA, [ethylenebis(oxoethylenenitrilo)] tetraacetic acid; His, histidine; HP, hematoporphyrin IX; IMM, inner mitochondrial membrane; MBM^+, trimethylammonium monobromobimane; MOPS, 4-morpholinepropanesulfonic acid; $^1O_2$, singlet oxygen; OMM, outer mitochondrial membrane; PhAsO, phenylarsine oxide; PT, permeability transition; PTP, permeability transition pore; RCR, respiratory control ratio; ROS, reactive oxygen species; TEM, transmission electron microscopy; VDAC, voltage-dependent anion channel

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histidine (His) residues located on the matrix side of the inner membrane make the pore responsive to pH (matrix acidification favoring pore closure through His protonation) [13]. A voltage sensor which is influenced by various effectors is proposed to make the pore opening sensitive to $\Delta\Psi_m$ (depolarization favoring pore opening) [14].

A major role in tuning the voltage sensor of the PTP is played by the oxidation-reduction state of vicinal thiols in cysteinyl (Cys) residues. Disulfide formation (as well as dithiol cross-linking) at a critical site (dubbed the "S-site") located in the matrix is associated with a higher probability of PT activation through a shift of the threshold potential at which pore opening occurs [15,16]. In addition, a class of pore-regulating thiols, which are sensitive to membrane-impermeant reagents (external thiols) has been also described. External thiols could be either located on the outer surface of the inner membrane, or on intermembrane/outer membrane regulatory protein(s), possibly at contact sites [17].

In 1997, Salet et al. [18] used a novel methodological approach to explore the regulatory properties of PTP-critical sites. Their method took advantage of the ability of porphyrins to bind to mitochondria and produce reactive oxygen species [ROS, mainly singlet oxygen ($^1O_2$)] upon irradiation with UV/visible light [19]. The $^1O_2$ transient photogenerated *in situ* can easily oxidize electron-rich protein targets, including Cys and His, provided that the substrate and the sensitizer are situated in close proximity [20,21]. Using the porphyrin photosensitizing properties, Salet et al. [18] demonstrated that hematoporphyrin (HP) localizes to strategic matrix sites containing PTP-regulating His. In the first example of PTP desensitization by ROS, His degradation by $^1O_2$ generated by vicinal photoactivated HP maintained the pore in a closed conformation, in sharp contrast with what is generally observed with oxidizing agents (see also refs. [22,23]).

Herein we find that pore inhibition following His photodegradation can be ascribed to a drop of reactivity of the internal PTP-activating thiols (the "S" site), which are no longer reactive with cross-linkers and oxidants. We have exploited this inactivation of the "S" site to study the contribution to PTP regulation of an additional class of sulfhydryls, which can also trigger the PT after oxidation or reaction with cross-linkers. Based on the PT-desensitizing effect of membrane-impermeant, thiol-protective reagents and on the PT-activating effect of copper-*o*-phenanthroline, we conclude that this group of thiols is located on the external side of the inner membrane.

2. Materials and methods

2.1. Reagents

Hematoporphyrin IX (HP) was obtained from Frontier Scientific (Logan, UT, USA). Stock solutions were prepared in dimethylsulfoxide (DMSO). Trimethylammonium monobromobimane (MBM^+) was a product of Calbiochem, UK. Copper-*o*-phenanthroline [$Cu(OP)_2$] was prepared just before use by mixing $CuSO_4$ with *o*-phenanthroline in a molar ratio of 1:2 in bidistilled water. All chemicals were of the highest purity commercially available.

2.2. Preparation of mitochondria

Liver mitochondria from Wistar rats were prepared by standard differential centrifugation. The final pellet was suspended in 0.25 M sucrose to give a protein concentration of 80–100 mg/ml, as measured by the biuret method. The functionality of mitochondria was established by measuring the respiratory control ratio (RCR) between the rate of oxygen consumption in state 3 (in the presence of 0.3 mM ADP) and in state 4 (in the absence of ADP) in a thermostatted ($T = 25$ °C), water-jacketed vessel, using a Clark electrode connected to a recorder. The incubation buffer contained 100 mM sucrose, 50 mM KCl, 10 mM Tris–Mops, 10 mM KH_2P0_4, 2 mM $MgCl_2$, 1 mM EDTA, 2 μM rotenone, pH 7.4. Succinate (5 mM) was used as the energizing substrate. Only mitochondrial suspensions with a RCR ≥ 3.0 were used.

2.3. Mitochondrial permeability transition

Mitochondrial PT was induced at 25 °C in a medium containing 200 mM sucrose, 10 mM Tris–Mops, 5 mM succinate, 1 mM P_i, 10 μM EGTA–Tris, 0.5 μg/ml oligomycin, 2 μM rotenone, pH 7.4 (standard medium) [14]. Ca^{2+}, phenylarsine oxide (PhAsO) and copper-*o*-phenanthroline [$Cu(OP)_2$] were used as PT inducers.

PT-induced osmotic swelling of mitochondrial suspensions was followed as the decrease in 90° light scattering at 540 nm, measured with a Perkin-Elmer LS 50 spectrophotofluorimeter [22].

The calcium retention capacity (CRC), i.e., the amount of Ca^{2+} accumulated and retained by mitochondria before the occurrence of the PT [24], was measured with a Ca^{2+}-selective electrode (Elektrode ISE Calcium, Crison Instruments, Barcelona, Spain).

2.4. Photosensitization of HP-labelled mitochondria

Mitochondria (0.5 mg/ml) were incubated for 1–2 min in the dark with the desired concentration of HP and then irradiated at 365 nm in a thermostated glass reaction vessel with a Philips HPW 125-W lamp (Philips, Eindhoven, The Netherlands). The fluence rate at the level of the preparations (40 W/m^2) was measured with a calibrated quantum-photo-radiometer (Delta OHM HD 9021). All irradiations were performed at 25 °C under magnetic stirring. Proper controls were carried out indicating that neither incubation with the photosensitizer in the dark nor illumination under identical experimental conditions but in the absence of porphyrin produced any appreciable changes in the parameters under study.

2.5. Transmission electron microscopy (TEM) of mitochondria

Mitochondria in the various experimental conditions were fixed for 30 min at 4 °C using glutaraldehyde at a final concentration of 1.5% (V/V) in 0.1 M cacodilate buffer (pH 7.2), then post-fixed with 1% OsO_4. Thin sections (60–80 nm) were stained with uranyl acetate in alcohol (50%) and lead citrate. Observations were made by using a FEI Tecnai F12 transmission electron microscope.

3. Results and discussion

3.1. Inhibition of the mitochondrial PT by HP-mediated photooxidative stress. Degradation of PTP-regulating His

We studied the effects of photoactivated HP on mitochondrial Ca^{2+} uptake and membrane permeabilization to sucrose. Mitochondria labelled with 3 μM HP were incubated for 2 min at 25 °C and the PT was triggered by Ca^{2+} after irradiation for increasing times at a fluence rate of 40 W/m^2. Non-irradiated mitochondria did not retain a 80 μM Ca^{2+} pulse, and a fast process of Ca^{2+} release readily followed the initial phase of Ca^{2+} uptake (Fig. 1A, trace a). This fast process of Ca^{2+} release was due to the opening of the PTP since it was accompanied by swelling (Fig. 1B, trace a), and the Ca^{2+} threshold was more than doubled in the presence of 1 μM CsA (not shown). After 20 s of irradiation (total light dose = 0.08 J/cm^2) mitochondria readily accumulated the initial 80 μM Ca^{2+} pulse, whereas Ca^{2+} efflux was triggered by a further 60 μM Ca^{2+} load (Fig. 1A, trace b) and the rate of membrane permeabilization was significantly decreased (Fig. 1B, trace b). After irradiation for 45 s (total light dose = 0.18 J/cm^2), the ability of mitochondria to take up and retain Ca^{2+} was further improved (Fig. 1A, trace c), and the inner mitochondrial membrane became impermeable to sucrose (Fig. 1B, trace c).

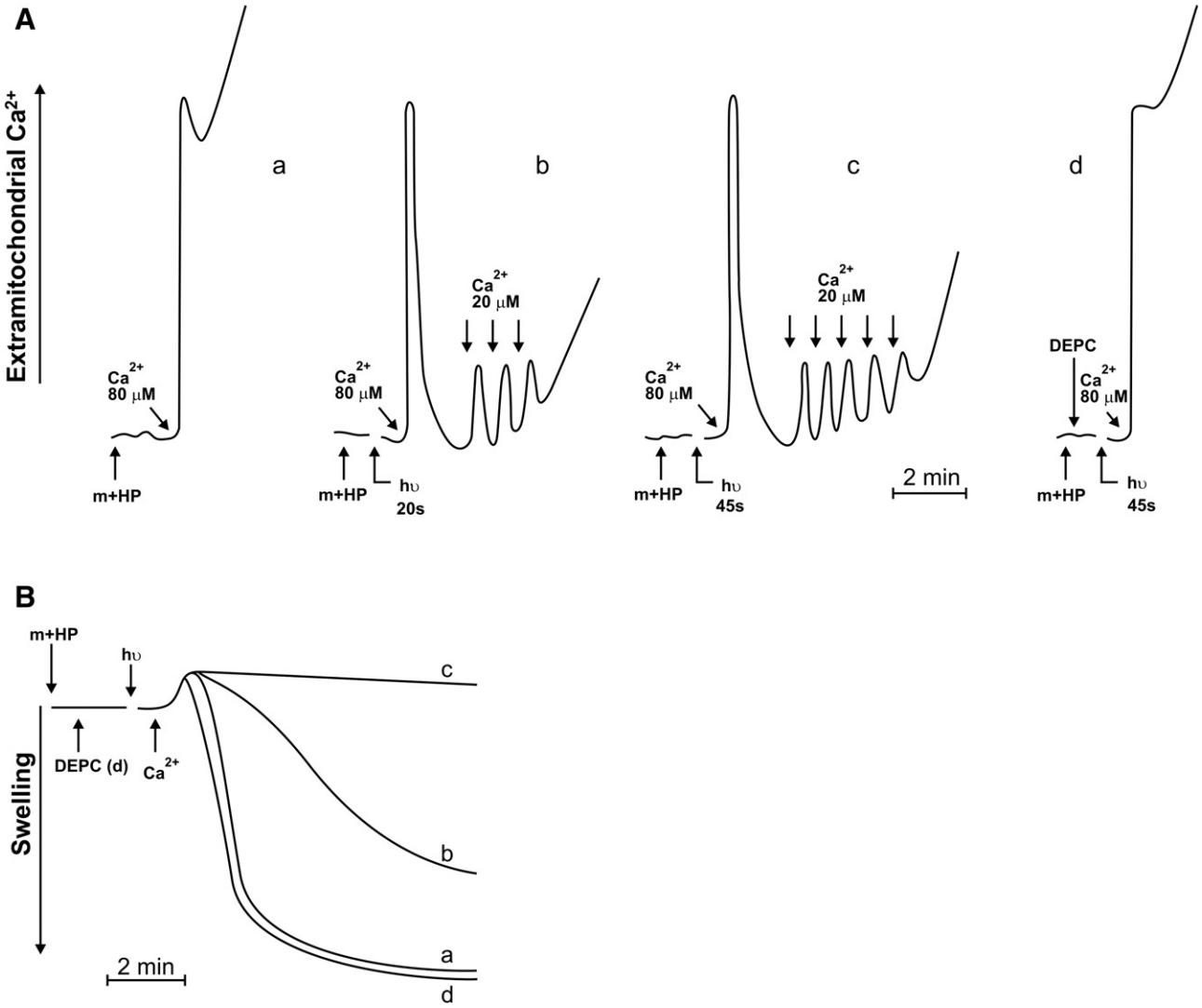

Fig. 1. Progressive mitochondrial PT inhibition at increasing irradiation times in HP-loaded mitochondria, as followed by the changes in PT-induced Ca^{2+} release (A) and matrix swelling (B). Mitochondria (0.5 mg/ml) labelled with HP (3 μM) (m + HP) were incubated for 2 min at 25 °C in the standard medium. Irradiation (hν) was performed for the indicated periods of time at a fluence rate of 40 W/m^2. Where indicated (trace d) mitochondria were supplemented with DEPC (200 μM). Panel A: Ca^{2+} pulses were added to the mitochondrial suspensions as indicated by arrows. Extramitochondrial Ca^{2+} was monitored by a Ca^{2+}-selective electrode. Panel B: the PT was triggered by 80 μM Ca^{2+}. Matrix swelling was followed as the decrease of 90° light scattering at 540 nm.

Maintenance of the pore in the closed state after mitochondrial irradiation in the presence of HP was previously attributed to photodegradation of matrix-exposed, PTP-regulating His residues [18,22,23]. Involvement of His as the photosensitive substrate is demonstrated by the antagonizing effects of pretreatment of mitochondria with diethyl pyrocarbonate (DEPC), which prevented the effects of photoirradiation (Fig. 1A and B, traces d). DEPC reacts with His, thereby hindering the addition of $^1O_2$ to the C_2–C_3 bond of the imidazole ring and its irreversible degradation [23].

3.2. Functional state of PTP-regulating thiols after His photodegradation. Unmasking external thiols

Under conditions leading to PT inhibition HP-mediated photodamage was selective to His, and PTP opening could still be triggered through other critical sites of the pore. As an example, the experiments reported in Fig. 2 show that non-irradiated mitochondria were permeabilized to sucrose by 80 μM Ca^{2+} (trace a), whereas irradiated mitochondria maintained their permeability barrier when challenged with an identical Ca^{2+} load (trace b), yet underwent permeabilization and swelling upon addition of the dithiol cross-

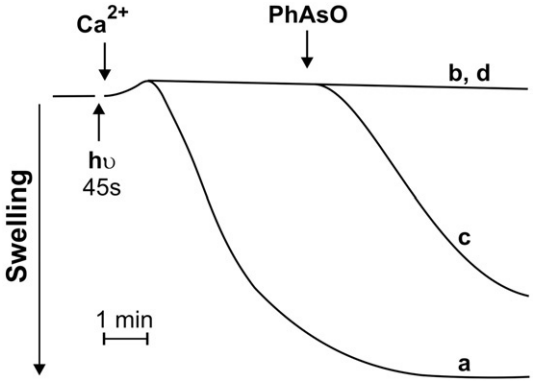

Fig. 2. Activation of the PT by PhAsO in HP-loaded, irradiated mitochondria. Trace a: the PT was induced by 80 μM Ca^{2+} in HP (3 μM)-labelled, non-irradiated mitochondria. Trace b: the Ca^{2+}-induced PT was inhibited by irradiation at 40 W/m^2 for 45 s (total light dose = 0.18 J/cm^2). Trace c: opening of the PTP was triggered in irradiated mitochondria by addition of 10 μM PhAsO. Trace d; 1 μM CsA was present in the incubation medium under the various experimental conditions. The PT trend was followed by the changes in 90° light scattering intensity at 540 nm, due to the changes of mitochondrial volume.

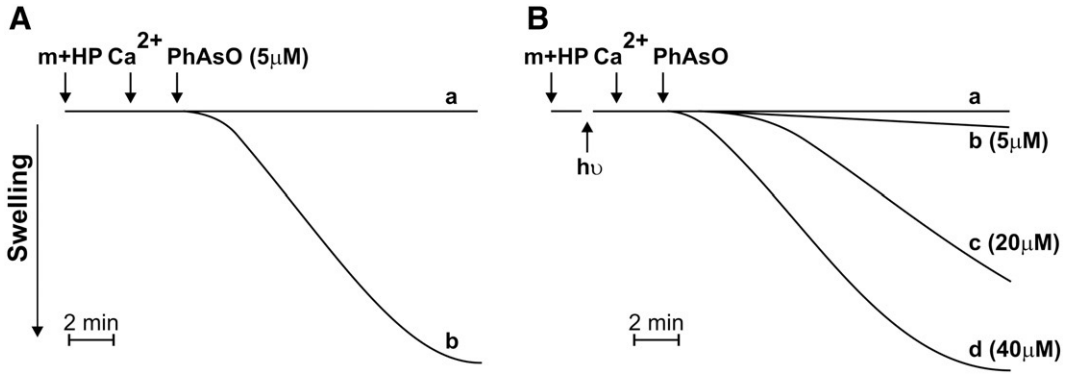

Fig. 3. Induction of the PT by PhAsO in HP-loaded, non-irradiated (A) and irradiated (B) mitochondria. Mitochondria (0.5 mg/ml) labelled with 3 μM HP, either in the dark (panel A) or irradiated for 45 s at 40 W/m^2 (total light dose = 0.18 J/cm^2) (panel B), were supplemented with a small Ca^{2+} pulse (5 μM), which was unable to induce PTP opening per se (panels A and B, traces a), followed by addition of PhAsO at concentration: 5 μM (panels A and B, traces b), 20 μM (panel B, trace c) or 40 μM (panel B, trace d). The PT was followed by the changes in 90° light scattering intensity at 540 nm.

linker PhAsO (trace c), in a process that maintained full sensitivity to CsA (trace d). These findings indicate that the pore remained competent for opening through critical thiols, which must have maintained the reduced state during the His photooxidation process (see also ref. [18]). Since photodamage is strictly localized to substrates that are in close proximity to the sensitizer, it follows that the PhAsO-reactive thiols and HP were beyond the critical distance (less than 0.02 μm, according to Moan and Berg, [20]) required for an efficient oxidative photoprocess (otherwise cysteine should be oxidized to cystine by $^1O_2$).

The –SH groups insensitive to the photoprocess could belong to the matrix-facing, critical cysteines ("S" site), as suggested by their availability to cross-linking reactions by the membrane-permeant PhAsO. Alternatively, His photodegradation could modify the pore-regulatory activity of the "S" site and lead to unmasking an additional class of thiols. The following set of experiments was aimed at discriminating between these two possibilities.

HP-labelled mitochondria, either non-irradiated (Fig. 3A) or exposed to a light dose of 0.18 J/cm^2 (40 W/m^2 for 45 s) (Fig. 3B), were supplemented with a small Ca^{2+} pulse (5 μM) unable to induce PTP opening per se (Fig. 3A, B, traces a), followed by PhAsO. In non-irradiated mitochondria, 5 μM PhAsO was sufficient to trigger the PT (Fig. 3A, trace b), a concentration that was instead ineffective in irradiated mitochondria (Fig. 3B, trace b). Remarkably, much higher PhAsO concentrations were necessary to induce swelling in irradiated mitochondria (Fig. 3B, traces c, d). Similar results were obtained with the membrane-permeant thiol oxidant diamide (DIA) (data not shown). These results suggest that the accessibility of PhAsO-reactive thiols was altered during irradiation, probably as a consequence of a conformational change brought about by His photooxidation [18,23].

To identify the class of thiols involved in the photoirradiation-dependent events we performed a set of experiments using DTT and MBM^+ as –SH protective agents. Monobromobimane (MBM) and the cationic derivative MBM^+ were introduced by Costantini et al. [25] as PTP-thiol reagents which neither inhibit the phosphate carrier, nor interfere with Ca^{2+} transport, energy coupling or ATP production and transport.

The process of PT activation by PhAsO (Fig. 4A, B, traces a) was prevented by treatment with 200 μM DTT in both non-irradiated (Fig. 4A, trace b) and irradiated (Fig. 4B, trace b) mitochondria. MBM^+ (up to 200 μM) was ineffective at blocking the DTT-sensitive site in non-irradiated mitochondria (Fig. 4A, trace c). Since this cationic species is impermeant to (or slowly permeating) cell membranes [26] we conclude that PhAsO interacted with internal thiols that could not be protected by MBM^+. In striking contrast, 10 μM MBM^+ significantly delayed opening of the pore in irradiated mitochondria (Fig. 4B, trace c). Protection of the PhAsO-reactive thiols by MBM^+ strongly suggests that in irradiated mitochondria PhAsO triggered the PT mainly *via* an external site. In keeping with this interpretation, addition of up to 0.5 mM MBM^+ to mitochondria did not cause any perturbation of the membrane potential as measured through the fluorescence changes of Pyronin G [22,23] (results not shown), in contrast to what observed

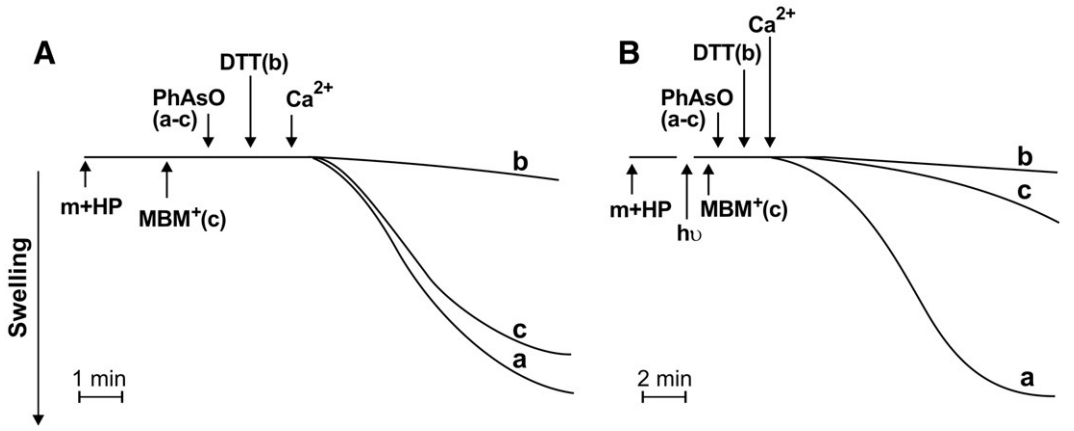

Fig. 4. Induction of the PT by PhAsO in HP-loaded, non-irradiated (A) and irradiated (B) mitochondria in the absence and in the presence of thiol-protective reagents. HP (3 μM)-labelled mitochondria (0.5 mg/ml), either non-irradiated (panel A) or irradiated for 45 s at 40 W/m^2 (total light dose = 0.18 J/cm^2) (panel B), were supplemented with 10 μM PhAsO, then 10 μM Ca^{2+} (a concentration not sufficient to induce PTP opening per se) was added (panels A and B, traces a). Where indicated, 200 μM DTT (panels A and B, traces b) or MBM^+ (10–200 μM in panel A and 10 μM in panel B, traces c) were also added. The PT was followed by the changes in 90° light scattering intensity at 540 nm.

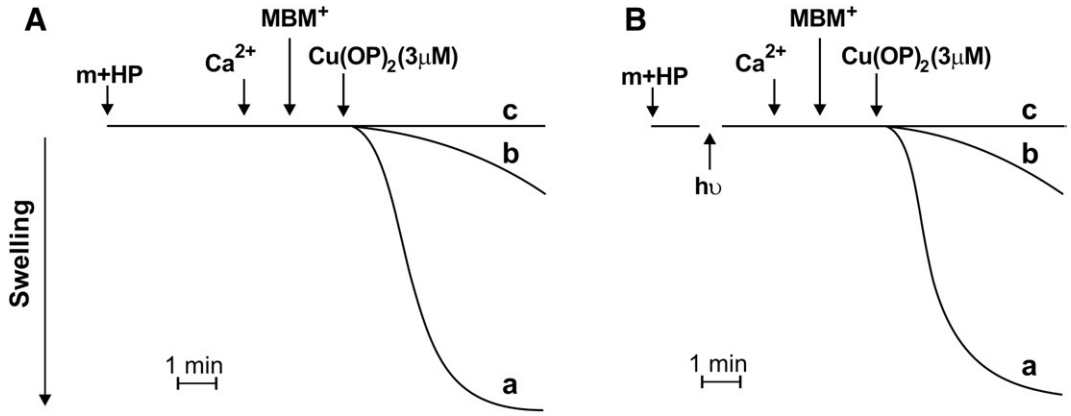

Fig. 5. Induction of the PT by $Cu(OP)_2$ in HP-loaded, non-irradiated (A) and irradiated (B) mitochondria in the absence and in the presence of the thiol-protective reagent, MBM^+. HP (3 μM)-labelled mitochondria (0.5 mg/ml), either non-irradiated (panel A) or irradiated for 45 s at 40 W/m^2 (total light dose = 0.18 J/cm^2) (panel B), were supplemented with 10 μM Ca^{2+} (a concentration not sufficient to induce PTP opening per se), then $Cu(OP)_2$ (3 μM) was added (panels A and B, traces a). Where indicated, 10 μM MBM^+ (panels A and B, traces b) were also added. In traces c, the various experiments were carried out in the presence of 1 μM CsA. The PT was followed by the changes in 90° light scattering intensity at 540 nm.

with Ca^{2+} [23] or with membrane-permeant lipophilic cations [27]. Thus, it appears that MBM^+ is not transported across the inner membrane; and that the MBM^+-inhibitable sites unmasked by irradiation are not located in the matrix, also based on the effects of membrane-impermeant $Cu(OP)_2$ (see below).

To further probe the involvement of external thiols in mitochondrial PT activation after His photooxidation, we used a PTP-triggering, membrane-impermeant reagent. In the next set of experiments HP-loaded mitochondria were assayed using the thiol oxidant $Cu(OP)_2$ as the PT inducer. It was previously shown that $Cu(OP)_2$ stimulates the PT by catalyzing the dithiol-disulfide interconversion of a class of external thiols [17]. Both non-irradiated (Fig. 5A) and irradiated (Fig. 5B) mitochondria underwent matrix swelling after exposure to 3 μM $Cu(OP)_2$ (Fig. 5A, B, traces a), in a process sensitive to CsA (Fig. 5A, B, traces c). In both cases, the effects of $Cu(OP)_2$ could be prevented by thiol reaction with MBM^+ (Fig. 5A, B, traces b). These results clearly indicate that PT induction and PT inhibition by MBM^+ in irradiated mitochondria was indeed due to the activation and protection of external thiols, respectively. These groups were not affected by any structural rearrangement after His photodegradation, because the concentration of $Cu(OP)_2$ required to induce the PT was identical in irradiated and non-irradiated mitochondria.

Taken together, these findings suggest that: (i) in non-irradiated mitochondria, pore opening *via* cross-linking of –SH groups by low PhAsO concentrations is mainly regulated by the internal, matrix-facing sites; (ii) His photodegradation causes a drop in reactivity of internal but not of external thiols, thus allowing to study the specific contribution of the latter to PT regulation. This class of thiols readily reacts with membrane-impermeants reagents, such as $Cu(OP)_2$, whereas more drastic conditions are necessary for the interaction with membrane-permeant compounds, such as PhAsO and DIA, which partition in both surface and internal mitochondrial membrane domains.

3.3. HP-mediated photooxidation of external thiols

The next set of experiments was aimed at determining whether HP-binding sites were also present near the external thiol domains, and could modify their reactivity upon irradiation. Since under the experimental conditions used thus far (3 μM HP, 45 s irradiation at 40 W/m^2) only HP-binding sites interfering with the critical His could be detected, we explored the effects of different times of irradiation and HP concentrations on the PT, as measured with the sensitive CRC assay (Fig. 6). These experiments revealed that the effect of irradiation was biphasic in all the experimental conditions used. Inhibition of the PT increased with the irradiation time up to a maximum, followed by a decreased inhibition at longer irradiation times. In all cases, HP-mediated photodamage causing PT inhibition could be ascribed to His photodegradation, as indicated by the counteracting effect of DEPC (results not shown). These findings suggest that the HP-binding sites adjacent to the critical His are highly selective for the porphyrin, being the most photovulnerable in the whole range of effective HP concentrations.

The CRC values rapidly decreased upon increase of the light dose. We found that the CRC decrease below control values could still be largely counteracted by CsA. As an example, Fig. 7 shows that 0.5 mg/ml of mitochondria incubated with 3 μM HP and supplemented with 40 μM Ca^{2+} (which does not affect the membrane permeability per se), then irradiated for 100 s at 40 W/m^2 (total light dose = 0.4 J/cm^2) underwent a large amplitude swelling (trace a) that was prevented by CsA (trace b). Similarly to most PTP inducers, HP + light needed Ca^{2+} as a permissive factor for PTP opening (see in trace c the lack of effect obtained in the absence of Ca^{2+}). Thus, in these protocols, PTP opening was due to a HP-dependent photooxidation process of a PTP-regulating site that is less photosensitive than His. This site coincides with the external critical thiols, as shown by the antagonizing effect of the membrane-impermeant thiol reagent MBM^+ (trace d). This result

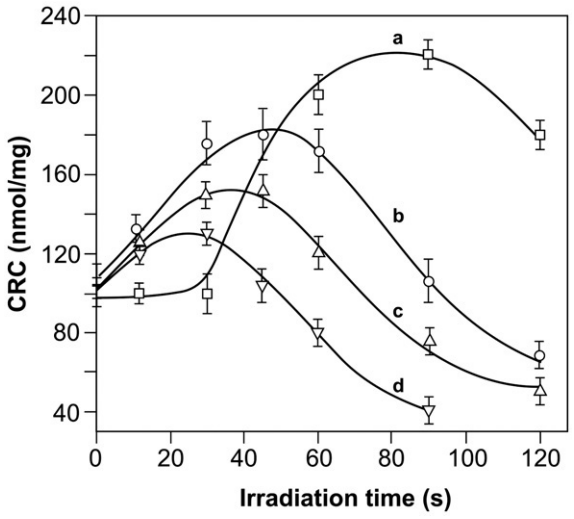

Fig. 6. Effects of irradiation on the PT in HP-loaded mitochondria at different HP concentrations and light doses. Mitochondria (0.5 mg/ml) were incubated for 2 min at 25 °C in the standard medium after labelling with: 1 μM (a), 3 μM (b), 4 μM (c), or 5 μM (d) HP. After irradiation for the indicated periods of time at a fluence rate of 40 W/m^2, mitochondria were loaded with a series of 10 μM Ca^{2+} pulses at 1 min intervals. PTP opening was determined as the CRC measured with a Ca^{2+}-selective electrode.

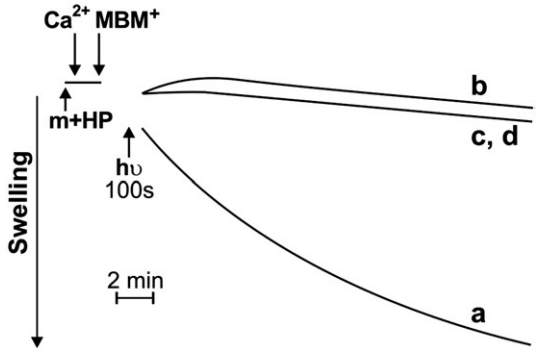

Fig. 7. Induction of mitochondrial PT after 100 s irradiation of HP-loaded mitochondria. HP (3 μM)-labelled mitochondria (0.5 mg/ml) after addition of 40 μM Ca^{2+} (a concentration not sufficient to induce PTP opening per se), were irradiated for 100 s at 40 W/m^2 (total light dose = 0.4 J/cm^2) (trace a). In trace b, 1 μM CsA was present in the incubation medium; in trace c, irradiation was performed in the absence of added Ca^{2+}; in trace d, 10 μM MBM^+ was added before irradiation. The PT was followed by the changes in 90° light scattering intensity at 540 nm.

is remarkable because it demonstrates that photooxidative stress mediated by the same sensitizer (HP) can either inhibit (through His degradation) or activate (through external thiol oxidation) the PT, depending on the interplay between light and sensitizer dose.

3.4. Effects of HP and irradiation on mitochondrial ultrastructure

We studied the effects of photooxidative events mediated by HP on mitochondrial ultrastructure, as analysed by TEM. Mitochondria maintained their integrity after loading with HP in the dark, as demonstrated by their regular shape with a well defined outer membrane and rich inner membrane infolding to define the cristae (Fig. 8A and B). Following PTP opening by 80 μM Ca^{2+}, mitochondria appeared swollen with decreased matrix electron density and increased volume (Fig. 8C). The mitochondrial membranes were well preserved when Ca^{2+} was added after exposure to 0.18 J/cm^2 light dose (40 W/m^2 for 45 s) (Fig. 8D), mitochondrial morphology being closely comparable to that obtained by pretreatment with 1 μM CsA (Fig. 8E).

HP-loaded mitochondria had normal morphology and folding of the inner membrane after exposure to 0.4 J/cm^2 light dose (40 W/m^2 for 100 s) in the absence of added Ca^{2+} (Fig. 8F) (i.e. a condition under which the PTP was in the closed state, Fig. 7). Addition of 40 μM Ca^{2+}, which induced PTP opening (Fig. 7), caused matrix swelling and expansion of the mitochondrial volume (Fig. 8G). These effects were prevented by pretreatment with 10 μM MBM^+ (Fig. 8H). It should be noted that the outer membrane could not be clearly detected after HP + light treatment when the PTP did not open (Fig. 8F and H); yet, the activity of monoaminooxidase was fully retained, indicating that impermeability barrier to proteins was fully maintained (data not shown). It appears likely that the observed structural reorganization is concomitant with the marked decrease of oxidative phosphorylation efficiency after prolonged irradiation times [18]. This would be consistent with the observations of Hackenbrock [28,29], which indicate that the internal mitochondrial structure is rather flexible, and linked to the metabolic state of the organelle.

4. Summary and conclusions

In photosensitization of biological materials by reactive oxygen species (including $^1O_2$) the photodamage is strictly limited to the immediate surroundings of the sensitizer because of the short diffusion distance and high reactivity of the photogenerated species. On this basis, selective targeting of photosensitive substrates located at or near the sensitizer binding sites is thus possible. This peculiar oxidation mechanism has provided more detailed information on residues regulating the PT than is possible to achieve with other

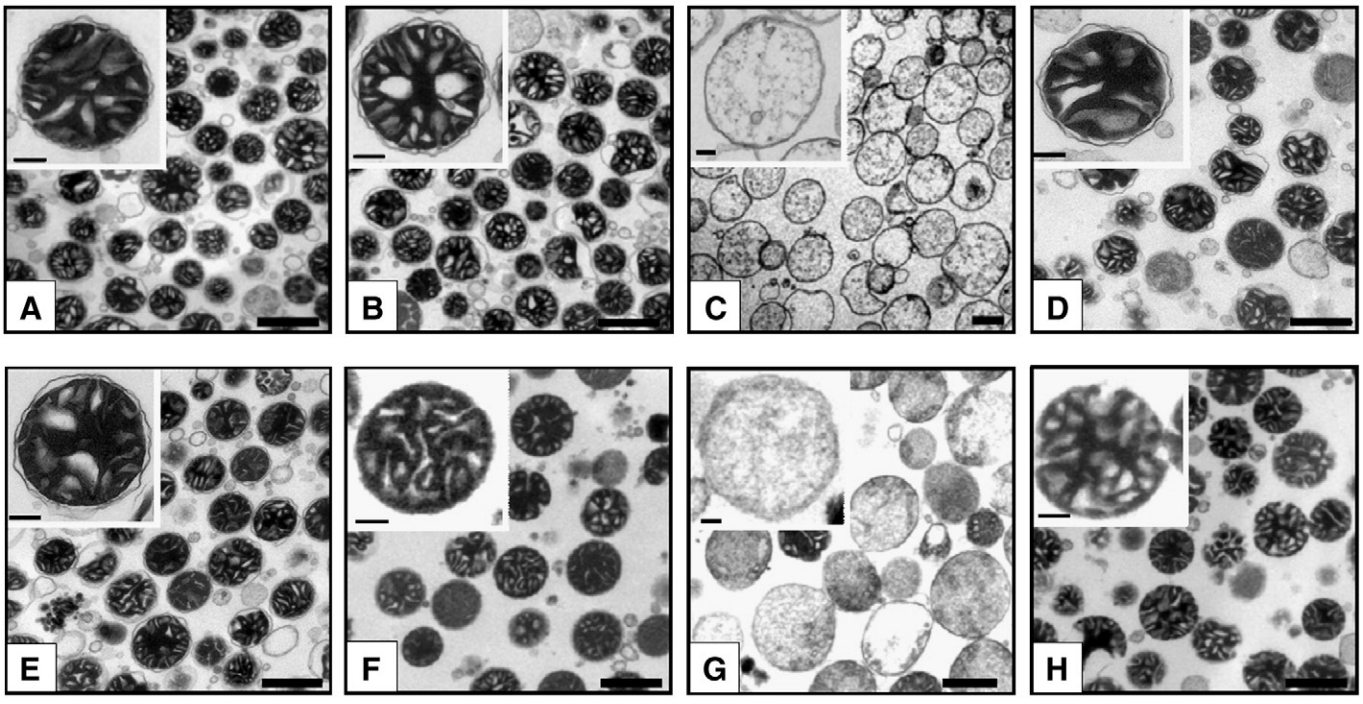

Fig. 8. Effects of HP loading and different light doses on mitochondrial ultrastructure. Mitochondria (0.5 mg/ml) were incubated for 2 min at 25 °C in the standard medium without (panel A) or with 3 μM HP (panels B–H). HP-loaded mitochondria were treated as follows: kept in the dark (panel B); supplemented with 80 μM Ca^{2+} to induce PTP opening (panel C); exposed to 0.18 J/cm^2 light dose (40 W/m^2 for 45 s) before addition of 80 μM Ca^{2+} (panel D); pretreated with 1 μM CsA before addition of 80 μM Ca^{2+} (panel E); exposed to 0.4 J/cm^2 light dose (40 W/m^2 for 100 s) in the absence of added Ca^{2+} (panel F); supplemented with 40 μM Ca^{2+} before exposure to 0.4 J/cm^2 light dose (panel G); supplemented with 40 μM Ca^{2+} plus 10 μM MBM^+ before exposure to 0.4 J/cm^2 light dose (panel H). Bars correspond to 1 μm (main figure) or 0.2 μm (insets).

oxidizing agents or conditions. In this study, whose results are summarized in Fig. 9, we were able to characterize the PTP-modulating properties of two sites which exhibited different sensitivity towards oxidation by vicinal, photoactivated HP.

Under basal conditions (step 1) the PTP favors the closed conformation, and the presence of HP does not affect the reactivity and pore-modulating properties of internal (matrix-exposed) and external cysteines (for clarity, the latter are represented as located on the outer surface of the IMM); indeed, an increased probability of PTP opening can be easily elicited by complex formation with PhAsO (which reacts with both sites) or $Cu(OP)_2$, a membrane-impermeant reagent that only oxidizes the external site (step 2). Photoirradiation for short periods of time hits the most photovulnerable site, which comprises matrix-exposed His (see also ref. [18]), and causes a secondary drop of reactivity of internal, pore-activating Cys, thus stabilizing the PTP in the closed conformation (step 3); indeed, matrix Cys can no longer react with PhAsO, probably because of a conformational rearrangement of the "S" site which makes these residues distant or poorly accessible to reagents. On this basis, the key His residues appear to play a role in PT activation as modulators of the conformation of internal thiol domains. In agreement, literature data indicate that protonation of the key His is associated with less extensive thiol oxidation [30] or cross-linking [23]. Thus, it appears that any modification of the His site interferes with the activity of the "S" site, suggesting that they are closely related both structurally and functionally.

The functional inactivation of internal Cys by His photodegradation in turn allows to study the role of external regulatory Cys, which can still undergo oxidation by $Cu(OP)_2$ (or complex formation by PhAsO, omitted for clarity) and thus increase the probability of PTP opening (step 4). Finally, photoirradiation for times longer than necessary to oxidize His causes direct oxidation of the external Cys, which is followed by PTP opening (step 5).

In conclusion, oxidative stress mediated by $^1O_2$ photogenerated in the presence of HP can either trigger or inhibit the mitochondrial PT depending on porphyrin localization and nature of the photosubstrate. The importance of the sensitizer binding site for the effects of $^1O_2$ on the PT was already demonstrated by comparative studies with two structurally different photoactivated dyes, HP (PT inhibition) and 4,5′,8-trimethylpsoralen (PT induction) [21]. In addition, the present data demonstrate that the photoprocess stimulated by the same sensitizer can switch the mitochondrial PT from inhibition to activation by a fine tuning of light intensity/sensitizer concentration combinations.

The identity of the mitochondrial proteins which bind the two PTP-regulating, photosensitive substrates remains undefined. Yet, some indications could arise from previous studies of mitochondrial function under irradiation. At increasing light doses, oxidative phosphorylation was the first function to be lost, whereas respiration, Ca^{2+} cycling, OMM-, matrix- and intermembrane-enzyme activities were more resistant in this order. Among the identified $^1O_2$-targets of the IMM, ANT inactivation was largely responsible for the decline of oxidative phosphorylation at short irradiation times [18,31]. Thus, it is tempting to speculate that ANT domains exposed to the matrix side might contain the highly photosensitive, critical His. Such His location would also agree with the desensitizing effect caused by His photodegradation on the oxidation of the vicinal, internal thiol groups if these are exposed to the matrix side of the ANT as well, as suggested by some authors [3,32–36]. This interpretation would be in agreement with a PT-regulatory role of ANT, but of course PTP photoinactivation may well involve other critical targets of the phosphorylation cycle, including the P_i carrier, which was recently postulated to contain PTP-critical thiol groups [37]. Work aimed at

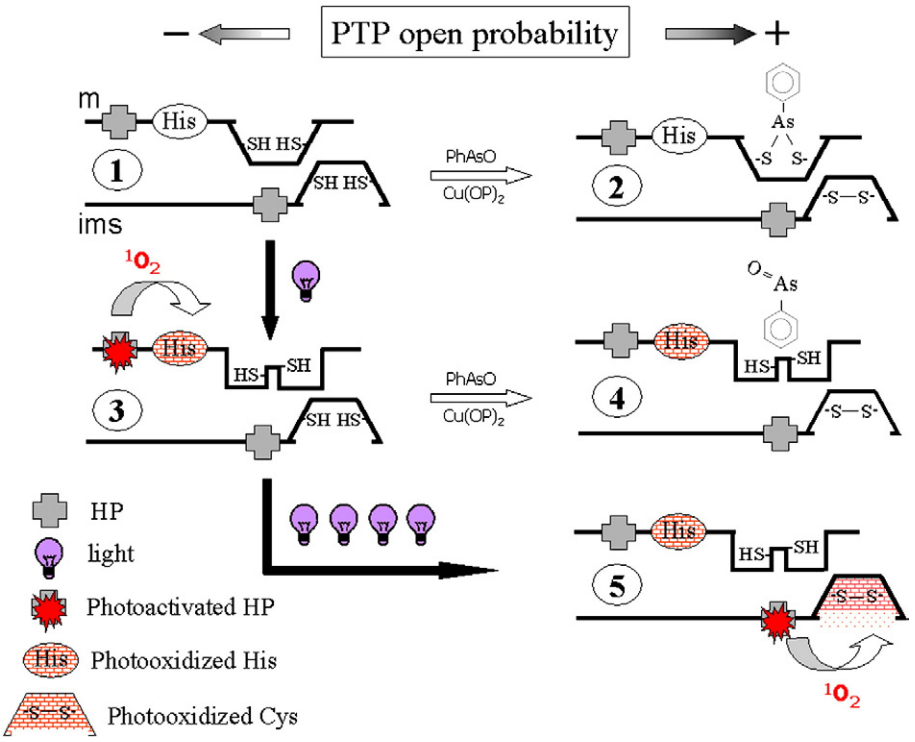

Fig. 9. Summary of the photodynamic events mediated by HP at PTP-regulating His and Cys residues. Matrix-facing (m) IMM HP-binding sites are located in close proximity to critical His residues. Additional HP-binding sites are adjacent to external, critical thiols. For clarity, external thiols are represented as located on the outer surface of the inner membrane facing the intermembrane space (ims). In the dark, HP does not affect the structural properties of His- and Cys-containing domains (step 1) and PTP can be opened through selective activation of internal or external Cys when low concentrations of PhAsO or $Cu(OP)_2$ are used (step 2; reaction of external Cys with PhAsO is not shown for clarity). After mitochondrial irradiation with moderate light doses, m-located photoactivated HP generates $^1O_2$. The photoprocess causes oxidation of the key His and a structural rearrangement of the internal thiol binding sites, which hinders the cross-linking reaction with PhAsO leading to PT inhibition (step 3). The PT can be reactivated through external thiols by oxidation with $Cu(OP)_2$ (step 4) or by cross-linking with PhAsO (not shown). Irradiation with high light doses causes photoactivation of ims-located HP leading to oxidation of ims-facing Cys by $^1O_2$ photogenerated *in situ* (step 5). For further explanation see text.

identifying the relevant protein targets of HP-mediated photooxidation is under way in our laboratories.

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