# Modulation of the Mitochondrial Cyclosporin A-Sensitive Permeability Transition Pore by Matrix pH. Evidence That the Pore Open–Closed Probability Is Regulated by Reversible Histidine Protonation†

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ABSTRACT: Energized mitochondria in sucrose medium take up a $Ca^{2+}$ pulse but do not show opening of the permeability transition pore (MTP) upon membrane depolarization by uncoupler. This is due to locking of the pore in the closed conformation by matrix acidification and fast $Ca^{2+}$ efflux following membrane depolarization (Petronilli, V., Cola, C., & Bernardi P. (1993) *J. Biol. Chem. 268*, 1011–1016). Here we show that addition of diethyl pyrocarbonate (DPC) prior to membrane depolarization restores the ability of uncoupler to induce MTP opening. Since DPC does not modify the rate and extent of matrix acidification and the rate and extent of $Ca^{2+}$ release following addition of uncoupler, its effects on pore opening appear to be due to modification of histidyl residues regulating the pore open–closed probability. This hypothesis was confirmed in studies with deenergized mitochondria incubated in potassium thiocyanate medium. While at acidic pH values pore opening is otherwise prevented, DPC allows $Ca^{2+}$-dependent pore opening at pH 6.5 in a process that maintains full sensitivity to cyclosporin A. Pore induction by DPC can be completely prevented and partially reversed by hydroxylamine, indicating that the effect of DPC can be specifically traced to carbethoxylation of histidyl residue(s) rather than to reaction with tyrosyl or sulfhydryl groups, while the possible involvement of lysyl residues cannot be excluded. Since DPC increases the pore open probability even at matrix pH values between 7.0 and 7.7, we propose that reversible protonation of one or more histidyl residues on the matrix side of the MTP plays a role in the physiological modulation of pore opening.

Mammalian mitochondria in vitro can easily undergo a $Ca^{2+}$-dependent permeability increase [reviewed by Gunter and Pfeiffer (1990)] due to opening of an unselective pore with a minimum diameter of 2.8 nm (Massari & Azzone, 1972), the mitochondrial permeability transition pore (MTP)[1] hypothesized in the pioneering studies of Hunter and Haworth (Haworth & Hunter, 1979; Hunter & Haworth, 1979a,b). Recent work from our laboratory suggests that the MTP coincides with the mitochondrial megachannel (MMC) previously identified at the single-channel level in rat liver mitoplasts (Petronilli et al., 1989). Indeed, both the MTP (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989) and the MMC (Szabò & Zoratti, 1991) are specifically blocked by submicromolar concentrations of cyclosporin A and respond in the same way and approximately in the same concentration range to most known effectors (Bernardi et al., 1992a,b; Szabò et al., 1992).

The MTP allows diffusion of solutes with molecular masses up to 1200 Da, and its opening is therefore not compatible with maintenance of ionic gradients. Since a low membrane permeability to anions and cations generally is a fundamental event in chemiosmotic energy conservation (Mitchell, 1961, 1966), pore opening has been widely considered as a potential threat to mitochondrial integrity, as testified by decades of mitochondrial research referring to the $Ca^{2+}$-dependent permeability increase as a membrane damaging event [see Gunter and Pfeiffer (1990) for a review]. This may not be the case, however, and our recent results suggest that the pore could be a regulated, voltage-gated channel which opens at low membrane potentials (Bernardi, 1992). The pore appears to be modulated by matrix pH (Bernardi et al., 1992a; Szabò et al., 1992; Bernardi, 1992) and by $Me^{2+}$ occupancy of two discrete sites for divalent cations with opposing effects on the pore open–closed probability (Bernardi et al., 1993; Petronilli et al., 1993).

In this paper we have investigated the mechanism by which the pore is regulated by matrix pH, following our previous observation that the MTP appears to be modulated in the range of $pH_i$ 6.5–8.0, with a steep decrease of the open probability (pore closure) as matrix pH decreases below 7.0 (Bernardi et al., 1992a; Szabò et al., 1992). We show that treatment of intact mitochondria with diethyl pyrocarbonate (DPC) allows MTP opening under conditions where the pore would otherwise be locked in the closed conformation by an acidic matrix pH. Pore opening by DPC is not due to direct modifications of matrix pH or of matrix $Ca^{2+}$ and, importantly, can be completely prevented and partially reversed by hydroxylamine. This indicates that the effect of DPC can be specifically traced to carbethoxylation of histidyl residue(s) rather than to reaction with tyrosyl or sulfhydryl groups, while the possible involvment of lysyl residues cannot be excluded (Miles, 1977). Since DPC increases the pore open probability even at matrix pH values between 7.0 and 7.7, we propose that reversible protonation of one or more histidyl residues on the matrix side of the MTP plays a role in the physiological modulation of pore opening.

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[1] Abbreviations: MTP, mitochondrial permeability transition pore; DPC, diethyl pyrocarbonate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein.

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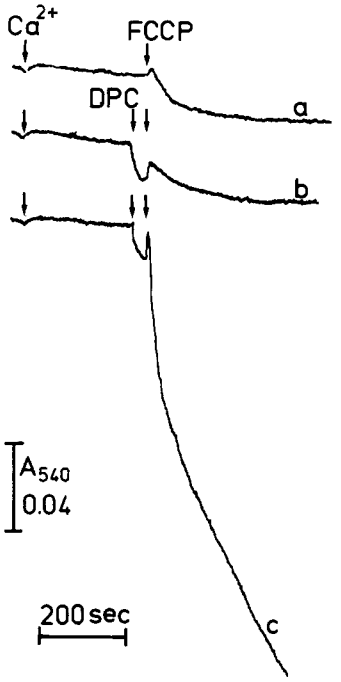


FIGURE 1: Effect of diethyl pyrocarbonate on mitochondrial permeability changes induced by depolarization in sucrose medium. The incubation medium contained 0.2 M sucrose, 10 mM Tris–Mops, pH 7.4, 5 mM succinate–Tris, 20 $\mu$M EGTA–Tris, 2 $\mu$M rotenone, and 1 $\mu$g mL$^{-1}$ oligomycin. In trace b, 0.4 $\mu$M cyclosporin A was present. Final volume was 2 mL; 25 °C. The experiments were started by the addition of 1 mg of mitochondria (not shown). Where indicated (arrows), 40 $\mu$M $Ca^{2+}$, 0.2 mM diethyl pyrocarbonate (DPC), and 0.2 $\mu$M FCCP were added.

## MATERIALS AND METHODS

Preparation of rat liver mitochondria and measurements of oxygen consumption were performed as described previously (Bernardi & Azzone, 1983). Mitochondrial $Ca^{2+}$ fluxes were determined from the absorbance changes of the metallochromic indicator Arsenazo III with an Aminco DW 2a dual-wavelength spectrophotometer as described (Bernardi et al., 1984). Mitochondrial volume changes were determined either from the absorbance changes at 540 nm as described (Bernardi et al., 1992a) or from the changes of 90° light scattering at 545 nm (Hunter & Haworth, 1979a) with a Perkin-Elmer 650-40 spectrofluorimeter equipped with magnetic stirring and thermostatic control. Mitochondrial loading with BCECF and $pH_i$ determinations were carried out exactly as described previously (Bernardi et al., 1992a). Measurements of membrane potential were carried out with a $TPMP^+$-selective electrode (initial $[TPMP^+]_o = 4$ $\mu$M) as described by Zoratti et al. (1986). The DPC stock solution (Sigma, St. Louis, MO) was diluted in anhydrous ethanol immediately prior to use. All chemicals were of the highest purity commercially available, while cyclosporin A was a generous gift of Sandoz Pharma AG (Basel).

## RESULTS

In the experiments depicted in Figure 1, mitochondria oxidizing succinate were incubated in a sucrose-based medium containing 20 $\mu$M EGTA. After volume equilibration, followed here as the absorbance of the mitochondrial suspension at 540 nm, a 40 $\mu$M $Ca^{2+}$ pulse was added, followed by 0.2 $\mu$M FCCP (trace a). As expected, addition of uncoupler was not followed by opening of the MTP. This is due to the intense matrix acidification accompanying $Ca^{2+}$ efflux upon addition of FCCP (Petronilli et al., 1993). If 0.2 mM histidine-specific reagent DPC was added after $Ca^{2+}$ accumulation and prior to uncoupler, however, addition of FCCP was immediately followed by a fast and extensive process of absorbance decrease indicative of mitochondrial swelling (trace c). Since cyclosporin A inhibited the swelling process completely (trace b), the experiment indicates that DPC restores the ability of uncoupler to specifically induce MTP opening.

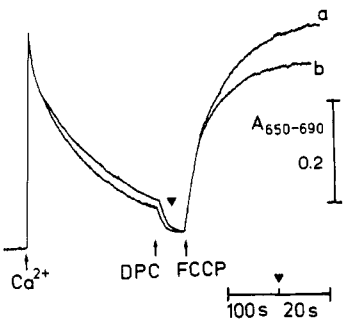


FIGURE 2: Effect of diethyl pyrocarbonate on mitochondrial $Ca^{2+}$ distribution and on depolarization-induced $Ca^{2+}$ efflux. The experimental conditions were as in Figure 1, except that 25 $\mu$M Arsenazo III was added. In trace b, 0.4 $\mu$M cyclosporin A was present. Where indicated (arrows), 40 $\mu$M $Ca^{2+}$, 0.2 mM diethyl pyrocarbonate (DPC), and 0.2 $\mu$M FCCP were added. Note the time scale change (▼).

Recent work from our laboratory has indicated that upon complete depolarization the pore open probability may be increased if the rate of $Ca^{2+}$ efflux via reverse uniport is decreased, e.g., by ruthenium red or by $P_i$ (Bernardi et al., 1993; Petronilli et al., 1993). We therefore tested whether DPC modifies the kinetics of uncoupler-induced $Ca^{2+}$ release. Figure 2 shows an experiment where extramitochondrial $Ca^{2+}$ fluxes were monitored with the metallochromic indicator Arsenazo III. After accumulation of a $Ca^{2+}$ pulse, addition of DPC was followed by a slight increase of $Ca^{2+}$ accumulation and by a fast process of $Ca^{2+}$ efflux upon addition of FCCP (trace a). Since under these conditions uncoupling is followed by pore opening (see trace c in Figure 1), we next assessed whether FCCP-induced $Ca^{2+}$ efflux was occurring through the uniporter or through the open pore. Figure 2, trace b, shows a similar experiment carried out in the presence of 0.4 $\mu$M cyclosporin A, i.e., under conditions where MTP opening was fully inhibited (see trace b in Figure 1). It can be seen that the initial rate of $Ca^{2+}$ efflux after addition of FCCP was indistinguishable from that observed in the absence of cyclosporin A. Thus, most of the FCCP-induced $Ca^{2+}$ efflux under these conditions is occurring via reverse uniport rather than through the pore. Since the rate of FCCP-induced $Ca^{2+}$ efflux in the absence of DPC was identical to that observed in the presence of DPC and cyclosporin A (not shown), then DPC restoration of the ability of FCCP to induce pore opening cannot be explained by a better retention of the accumulated $Ca^{2+}$.

A major determinant of the pore open probability in depolarized mitochondria is the absolute value of matrix pH, since the closed conformation is favored as matrix pH is decreased (Bernardi, 1992; Bernardi et al., 1992a; Szabò et al., 1992; Petronilli et al., 1993). The next question we asked was if the effect of DPC could be due to a direct buffering of matrix pH. Therefore, we carried out measurements of mitochondrial matrix pH in BCECF-loaded mitochondria since the effects of DPC on pore opening were not affected by BCECF loading (not shown). Figure 3 shows that the

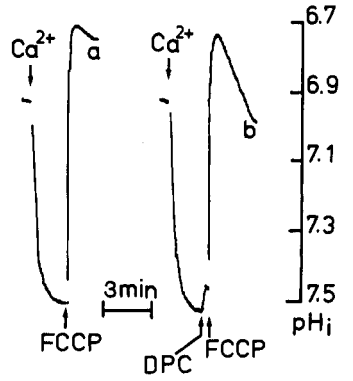


FIGURE 3: Effect of diethyl pyrocarbonate on matrix pH changes induced by $Ca^{2+}$ and depolarization. The experimental conditions were as in Figure 1. The experiments were started by the addition of 0.4 mg of BCECF-loaded mitochondria (not shown). Where indicated (arrows), 40 $\mu$M $Ca^{2+}$, 0.2 mM diethyl pyrocarbonate (DPC), and 0.2 $\mu$M FCCP were added.

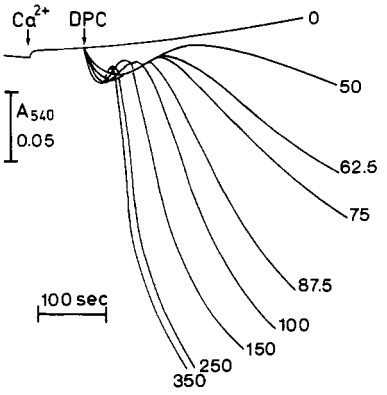


FIGURE 4: $Ca^{2+}$-dependent pore induction by diethyl pyrocarbonate in sucrose medium. Experimental conditions were as in Figure 1. The experiments were started by the addition of 1 mg of mitochondria (not shown). Where indicated (arrows), 40 $\mu$M $Ca^{2+}$ and the concentrations ($\mu$M) of diethyl pyrocarbonate (DPC) labeling each trace were added.

addition of $Ca^{2+}$ to respiring mitochondria was followed by matrix alkalinization, due to the $H^+$ extrusion accompanying $Ca^{2+}$ uptake, while addition of FCCP caused an intense matrix acidification (trace a). Addition of DPC prior to FCCP caused a negligible effect on matrix pH, while the acidification following addition of FCCP was identical to that observed in the absence of DPC (trace b). The only difference we could detect in the presence of DPC was a fast fluorescence increase (corresponding to apparent matrix alkalinization) after FCCP-induced acidification (trace b). This was due to BCECF leakage through the open pores of increasing numbers of mitochondria since this fluorescence increase was completely abolished by 0.4 $\mu$M cyclosporin A (not shown).

The experiments presented so far show that DPC allows opening of the MTP upon depolarization. Figure 4 shows that after accumulation of a $Ca^{2+}$ pulse, addition of DPC was followed by spontaneous pore opening after a lag phase. The rate of permeabilization became faster as the concentration of DPC was increased, while the lag phase was proportionally reduced. Thus, DPC behaved as a classical $Ca^{2+}$-dependent pore inducer even in the absence of added FCCP. This effect could be partly traced to an inhibition of the maximal rate of electron transfer by DPC, while a direct protonophoric effect could be ruled out by titrations of the state 4 respiratory rate with DPC (not shown). The inhibitory effect of DPC on respiration can be explained by the key role of histidine residues

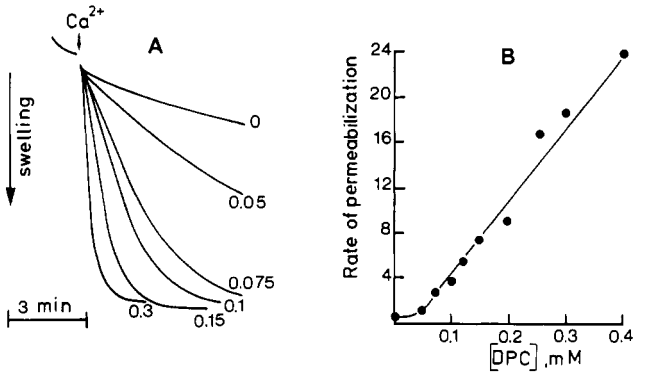


FIGURE 5: $Ca^{2+}$-dependent pore induction by diethyl pyrocarbonate in potassium thiocyanate medium at pH 6.5. The incubation medium contained 0.1 M KSCN, 5 mM Mops-KOH, pH 6.5, 20 $\mu$M EGTA-KOH, 2 $\mu$M rotenone, and 50 ng $mL^{-1}$ antimycin A. Final volume was 2 mL; 25 °C. The experiments were started by the addition of 1 mg of mitochondria (not shown), and the changes of 90° light scattering at 545 nm were recorded. Panel A: where indicated, 0.2 mM $Ca^{2+}$ was added in the presence of the concentrations (mM) of diethyl pyrocarbonate (DPC) labeling each trace. Panel B: values on the ordinate refer to the rate of mitochondrial permeabilization following $Ca^{2+}$ addition as a function of the concentration of diethyl pyrocarbonate (arbitrary units) from experiments similar to those depicted in panel A. For the sake of clarity panel A shows only representative recordings on a condensed time scale.

in electron-transfer reactions, particularly within cytochrome oxidase (Gennis, 1992).

The complexity of the effects of DPC on mitochondrial respiration and the fast changes of matrix pH and matrix $Ca^{2+}$ following addition of FCCP (Figures 2 and 3) make it extremely difficult to analyze the effects of DPC on pore opening at constant values of matrix pH in respiring mitochondria. We therefore assessed the effects of DPC in nonrespiring mitochondria incubated in KSCN medium. Under these conditions, diffusion of the lipophilic $SCN^-$ anion provides the driving force for electrophoretic $Ca^{2+}$ accumulation (Selwyn et al., 1970). $Ca^{2+}$ influx in turn collapses the $SCN^-$ diffusion potential and therefore prevents development of a large membrane potential which would counteract the increase of pore open probability afforded by matrix $Ca^{2+}$ (Bernardi et al., 1992b; Petronilli et al., 1993). Thus, since the permeability for $SCN^-$ and $Ca^{2+}$ is much higher than that for $H^+$, it is possible to achieve $Ca^{2+}$-dependent MTP opening in the absence of net $H^+$ movements.

Figure 5, panel A, shows an experiment where respiratory-inhibited mitochondria were incubated in a KSCN-based medium at pH 6.5. As expected, addition of $Ca^{2+}$ was not followed by MTP activation owing to the strong inhibitory effect of matrix $H^+$ on pore opening (Bernardi et al., 1992a; Szabò et al., 1992). However, if increasing concentrations of DPC were present, immediate MTP opening followed the addition of $Ca^{2+}$ (panel A), and the rate of permeabilization increased linearly with the concentration of DPC (panel B). It may be noted that the line fitting the experimental values is slightly shifted from the origin (panel B). This is most likely due to spontaneous DPC hydrolysis occurring between the addition of mitochondria and that of $Ca^{2+}$.

Figure 6 shows the effect of hydroxylamine on MTP induction by 0.1 mM DPC and 0.2 mM $Ca^{2+}$ in nonrespiring mitochondria in KSCN medium at pH 6.5. It can be seen that addition of increasing concentrations of hydroxylamine inhibited MTP opening in a dose-dependent fashion. A 45% inhibition could also be obtained within 4 min of the addition of 10 mM hydroxylamine after, rather than before, DPC (not shown). As discussed more in detail below, these experiments

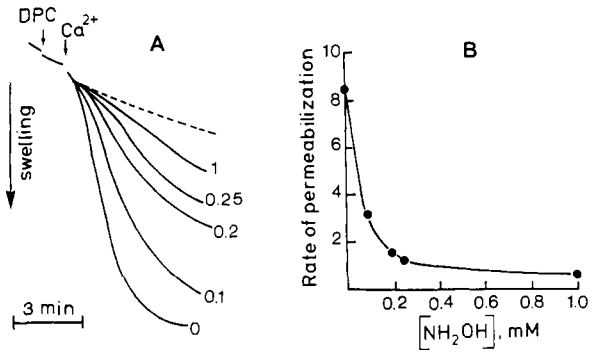


FIGURE 6: Inhibition of diethyl pyrocarbonate-dependent pore induction by hydroxylamine. Experimental conditions were as in Figure 5. Panel A: where indicated, 0.1 mM diethyl pyrocarbonate (DPC) and 0.2 mM $Ca^{2+}$ where added in the presence of the concentrations (mM) of hydroxylamine labeling each trace; in the dashed trace, no DPC or hydroxylamine was added. Panel B: values on the ordinate refer to the rate of mitochondrial permeabilization following $Ca^{2+}$ addition as a function of the concentration of hydroxylamine (arbitrary units).

strongly suggest that the effect of DPC can be specifically traced to carbethoxylation of histidyl residues rather than to reaction with tyrosyl or sulfhydryl groups, while a modification of lysyl residues cannot be ruled out unequivocally (Ovadi et al., 1967; Burstein et al., 1974; Miles, 1977; Papini et al., 1989).

The experiments presented so far indicate that preventing histidine protonation at acidic matrix pH values prevents pore inhibition by $H^+$. The last question we asked was whether this is also the case at neutral and alkaline matrix pH values. We have therefore carried out parallel measurements of matrix pH and of the rate of permeabilization induced by $Ca^{2+}$ in KSCN medium at varying external pH in BCECF-loaded mitochondria. Preliminary experiments indicated that matrix pH equilibration at alkaline values of external pH is extremely slow, with a continuous $pH_i$ drift which makes reliable correlations with the onset of pore opening difficult (not shown). Therefore, these experiments were performed on mitochondria aged in vitro, since we found that in aged mitochondria matrix pH equilibration was rapid. The results of Figure 7 show that these mitochondria were fully competent for pore induction by $Ca^{2+}$ and exhibited a $pH_i$ optimum of about 7.3 (circles). In the whole range of matrix pH measured, DPC greatly increased the rate of $Ca^{2+}$-dependent permeabilization, while the $pH_i$ optimum was unchanged (squares), and the permeabilization process maintained full sensitivity to cyclosporin A (diamonds). It is interesting that the rate of permeabilization declined as matrix pH was increased above 7.3, indicating that the pH optimum for pore opening is poised at matrix pH values close to those presumed to occur in vivo (Jung et al., 1989).

## DISCUSSION

In this paper we have shown that the histidine-selective reagent DPC is an inducer of the mitochondrial permeability transition pore. Indeed, addition of DPC to $Ca^{2+}$-loaded mitochondria determines spontaneous opening of the pore after a lag phase (Figure 4). Importantly, DPC has the ability to allow MTP opening under conditions (addition of uncoupler to respiring mitochondria in sucrose medium, Figures 1–3; addition of $Ca^{2+}$ to nonrespiring mitochondria in acidic thiocyanate medium, Figures 5 and 6) where the low matrix pH would otherwise lock the pore in the closed conformation despite deenergization (Bernardi, 1992; Bernardi et al., 1992a, 1993; Szabò et al., 1992; Petronilli et al., 1993).

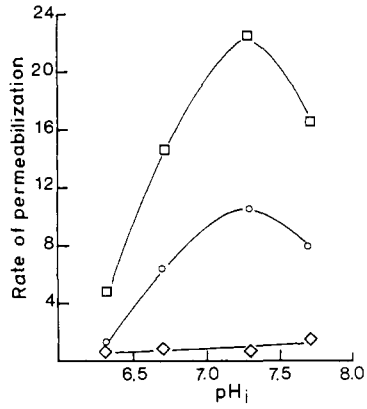


FIGURE 7: Effect of hydroxylamine and cyclosporin A on $Ca^{2+}$-dependent pore induction by diethyl pyrocarbonate at varying matrix pH. Experimental conditions were as in Figure 5, except that external pH was adjusted to 6.5, 7.0, 7.5, or 8.0 with KOH. Corresponding values of matrix pH, determined from BCECF fluorescence measurements, were 6.3, 6.7, 7.3, and 7.7, respectively. Circles, no further additions; squares, 75 $\mu$M diethyl pyrocarbonate; diamonds, 75 $\mu$M diethyl pyrocarbonate plus 0.4 $\mu$M cyclosporin A. The experiments were started by the addition of 0.4 mg of BCECF-loaded mitochondria followed by 0.2 mM $Ca^{2+}$. Values on the ordinate refer to the rate of permeabilization measured after $Ca^{2+}$ addition (arbitrary units) and are plotted against matrix pH.

Recent work from our laboratory suggests that the MTP may be a voltage-gated channel opening at low membrane potentials and regulated by matrix pH and by $Me^{2+}$ occupancy of two sites, one on the matrix side and one on the cytosolic side of the pore (Bernardi, 1992; Bernardi et al., 1992a,b, 1993; Szabò et al., 1992; Petronilli et al., 1993). We have shown that the pore open–closed probability may be modulated by these effectors (i.e., membrane potential, matrix pH, and $Me^{2+}$ ions) and that the apparent complexity of pore regulation by a variety of agents could be explained by their combinatorial effects on these few basic regulatory steps (Petronilli et al., 1993). In the light of these observations it is important to point out that the effects of DPC are due neither to a direct buffering of matrix pH nor to a modification of the mitochondrial $Ca^{2+}$ efflux rate. Since DPC is fully effective in deenergized mitochondria (either by addition of uncoupler in the presence of substrate or by addition of respiratory inhibitors), it also appears that its inhibitory effect on the maximal rate of electron transfer is not relevant for pore induction by DPC. Thus, DPC affects pore activity by a novel mechanism, most likely by modifying critical histidyl residues involved in the regulation of the pore open–closed transition.

DPC readily reacts with histidyl residues both in model systems and in isolated proteins to yield *N*-carbethoxyhistidyl derivatives [see Miles (1977) for a review]. In some cases, however, modification of other residues, including tyrosyl, sulfhydryl, and lysyl groups, has been reported (Miles, 1977, and references therein). The possibility that the effects of DPC may be mediated by reaction with sulfhydryl rather than histidyl residues is of particular concern, since a large amount of data indicates that MTP opening may be related to oxidation of disulfides [e.g., Le-Quoc and Le-Quoc (1982), Novgorodov et al. (1987), Chàvez and Holguìn (1988), Fagian et al. (1990), and Lenartowicz et al. (1991)]. Strong evidence against the involvement of sulfhydryl and tyrosyl residues comes from the observation that hydroxylamine prevents the effects of DPC on pore opening. On the other hand, addition of hydroxylamine after DPC leads only to partial inhibition of pore opening, leaving two interpretations open. The first possibility is that only histidyl groups have reacted, but reversal is incomplete because the time required for full decarbethox-

ylation by hydroxylamine is much longer than is possible to test in experiments with intact mitochondria. Indeed, reported reaction times vary between 30 min and 22 h, depending on the protein and the pH (Miles, 1977). In the case of purified fragment A of diphteria toxin, which contains only one histidine, decarbethoxylation required 2 h with 40 mM hydroxylamine and was not complete despite the demonstrable absence of modification of lysyl residues (Papini et al., 1989). The second possibility is that, besides histidine, one or more lysyl residues have been modified as well and that this modification in turn affects pore activity. Although we cannot rule out this possibility, we want to stress that the pH profile of pore modulation in the range 6.5–8.0 (Bernardi et al., 1992a) could easily be accounted for with the involvement of histidyl residues alone.

The data presented in this paper thus provide an attractive explanation for the observed effect of matrix pH on the pore: reversible protonation of one or more critical histidyl residues on the matrix side of the pore can regulate its open–closed transition. Since matrix pH is controlled by mitochondrial $H^+$, cation, anion, and substrate transport systems [e.g., Baysal et al. (1991), Brierley et al. (1991), and Jung et al. (1992)], these findings appear to support our proposal that the MTP is a highly regulated pathway likely to play a role in mitochondrial physiology. The increasing availability of pore-specific effectors and the increasing understanding of the key factors involved in pore regulation in vitro should soon allow a better understanding of its role(s) in vivo.

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## REFERENCES

Baysal, K., Brierley, G. P., Novgorodov, S., & Jung, D.W. (1991) *Arch. Biochem. Biophys. 291*, 383–389.

Bernardi, P. (1992) *J. Biol. Chem. 267*, 8334–8339.

Bernardi, P., & Azzone, G. F. (1983) *Biochim. Biophys. Acta 724*, 212–223.

Bernardi, P., Paradisi, V., Pozzan, T., & Azzone, G. F. (1984) *Biochemistry 23*, 1645–1651.

Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabò, I., & Zoratti, M. (1992a) *J. Biol. Chem. 267*, 2934–2939.

Bernardi, P., Zoratti, M., & Azzone, G. F. (1992b) in *Mechanics of Swelling: from Clays to Living Cells and Tissues* (Karalis, T.K., Ed.) pp 357–377, Springer-Verlag, Berlin.

Bernardi, P., Veronese, P., & Petronilli, V. (1993) *J. Biol. Chem. 268*, 1005–1010.

Brierley, G. P., Panzeter, E. S., & Jung, D. W. (1991) *Arch. Biochem. Biophys. 288*, 358–367.

Broekemeier, K. M., Dempsey, M. E., & Pfeiffer, D. R. (1989) *J. Biol. Chem. 264*, 7829–7830.

Chàvez, E., & Holguìn, J.A. (1988) *J. Biol. Chem. 263*, 3582–3587.

Crompton, M., Ellinger, H., & Costi, A. (1988) *Biochem. J. 255*, 357–360.

Fagian, M. M., Pereira-da-Silva, L., Martins, I. S., & Vercesi, A. E. (1990) *J. Biol. Chem. 265*, 19955–19960.

Fournier, N., Ducet, G., & Crevat, A. (1987) *J. Bioenerg. Biomembr. 19*, 297–303.

Gennis, R. B. (1992) *Biochim. Biophys. Acta 1101*, 184–187.

Gunter, T. E., & Pfeiffer, D. R. (1990) *Am. J. Physiol. 258*, C755–C786.

Haworth, R. A., & Hunter, D. R. (1979) *Arch. Biochem. Biophys. 195*, 460–467.

Hunter, D. R. & Haworth, R. A. (1979a) *Arch. Biochem. Biophys. 195*, 453–459.

Hunter, D. R., & Haworth, R. A. (1979b) *Arch. Biochem. Biophys. 195*, 468–477.

Jung, D. W., Davis, M. H., & Brierley, G. P. (1989) *Anal. Biochem. 178*, 348–354.

Jung, D. W., Apel, L. M., & Brierley, G. P. (1992) *Am. J. Physiol. 262*, C1047–C1055.

Lenartowicz, E., Bernardi, P., & Azzone, G. F. (1991) *J. Bioenerg. Biomembr. 23*, 679–688.

Le-Quoc, K., & Le-Quoc, D. (1982) *Arch. Biochem. Biophys. 216*, 639–651.

Massari, S., & Azzone, G. F. (1972) *Biochim. Biophys. Acta 283*, 23–29.

Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.

Mitchell, P. (1961) *Nature 191*, 144–148.

Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc. 41*, 445–501.

Novgorodov, S. A., Kultayeva, E. V., Yaguzinsky, L. S., & Lemeshko, V. V. (1987) *J. Bioenerg. Biomembr. 19*, 191–202.

Ovadi, J., Libor, S., & Elodi, P. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung. 2*, 455–458.

Papini, E., Schiavo, G., Sandonà, D., Rappuoli, R., & Montecucco, C. (1989) *J. Biol. Chem. 264*, 12385–12388.

Petronilli, V., Szabò, I., & Zoratti, M. (1989) *FEBS Lett. 259*, 137–143.

Petronilli, V., Cola, C., & Bernardi, P. (1993) *J. Biol. Chem. 268, 1011-1016.*

Selwyn, M. J., Dawson, A. P., & Dunnett, S. J. (1970) *FEBS Lett. 10*, 1–5.

Szabò, I., & Zoratti, M. (1991) *J. Biol. Chem. 266*, 3376–3379.

Szabò, I., Bernardi, P., & Zoratti, M. (1992) *J. Biol. Chem. 267*, 2940–2946.

Zoratti, M., Favaron, M., Pietrobon, D., & Azzone, G. F. (1986) *Biochemistry 25*, 760–767.