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Pseudosubstrate hydrolysis by the erythrocyte plasma membrane Ca^{2+}-ATPase: kinetic evidence for a modified E_1 conformation in dimethylsulfoxide

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Abstract

The purified Ca^{2+}-ATPase of pig red cells displays a phosphatase activity towards *p*-nitrophenylphosphate which is inhibited by Ca^{2+} in the absence of solvents, and activated by calmodulin. This activity has been attributed to the E_2 conformation of the enzyme. Here we show that the pNPPase activity in the absence of Ca^{2+} is stimulated 10–25-fold by the presence of the organic solvent dimethylsulfoxide (Me_2SO). This is an activation that surpasses by severalfold that induced by calmodulin in the absence of the solvent. At 30% Me_2SO, activation by calmodulin disappears. In the absence of calmodulin and at pH 7.2, the Ca^{2+} concentration needed for half-maximal inhibition of the pNPPase activity (K_i) increases from 130 μM in the absence of Me_2SO to 860 μM at 30% Me_2SO. This effect of Me_2SO is enhanced at pH 8.0: the K_i for Ca^{2+} increases from 2.7 μM in the absence of the solvent to 2.0 mM in its presence. However, the $K_{0.5}$ for Ca^{2+} activation of the ATPase activity decreases from 8.3 to 2.6 μM following addition of the same Me_2SO concentration. This indicates that, even in the presence of Me_2SO, μM Ca^{2+} concentrations shift the equilibrium towards E_1 but the decrease in activity that would be expected if pNPP hydrolysis were catalysed exclusively by the E_2 conformation is not observed. The affinity for pNPP as a substrate increases from 2.6 mM in the absence of Me_2SO to 1.6 mM in the presence of 20% Me_2SO. These results suggest that Me_2SO induces multiple effects in the Ca^{2+}-ATPase that: (i) increase the reactivity of E_2 towards substrate; (ii) surpass the activation by calmodulin and, (iii) allow the enzyme to hydrolyze pNPP even when Ca^{2+} is bound to the high-affinity sites of the enzyme. The change in reactivity is attributed to an increase on substrate catalysis rather than on pNPP binding.

Keywords: Erythrocyte plasma membrane Ca^{2+}-ATPase; pNPPase; Dimethylsulfoxide; Calmodulin

1. Introduction

P-type ATPases are a class of integral membrane enzymes that transport ions against their gradients using the free energy of ATP hydrolysis. All the models proposed for the P-type ATPases assume the existence of two main conformations during the catalytic cycle: E_1, which can be phosphorylated by

Abbreviations: pNPP, *p*-nitrophenylphosphate; pNP, *p*-nitrophenol; pNPPase, *p*-nitrophenylphosphatase; CaM, calmodulin; Me_2SO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid disodium salt dihydrate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PM Ca^{2+}-ATPase, plasma membrane Ca^{2+}-ATPase

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ATP, and E_2, which can be phosphorylated by P_i. The equilibrium between these two conformations is influenced mainly by the cation(s) to be transported. In the case of the PM Ca^{2+}-ATPases, the E_1 conformation is favored by binding of Ca^{2+} to the high-affinity intracellular sites, triggering phosphorylation by ATP.

A number of important features of the catalytic cycle of the P-type ATPases have been deduced from studies of the phosphatase activity of these enzymes. With the notable exception of the sarcoplasmic reticulum ATPase [1] and of the Na^+/K^+-ATPase at very low K^+ concentrations [2], the phosphatase activity has been attributed to the E_2 conformation, because it does not support cation transport and because the activity is inhibited by the ion to be transported [2,3]. In these enzymes the kinetic properties of the catalytic site in the E_2 conformation are greatly influenced by the medium hydrophobicity indicating that water plays an important role for the mechanism of catalysis of this conformer [4–6].

This report characterizes the catalytic cycle of *p*-nitrophenylphosphate hydrolysis by erythrocyte membrane Ca^{2+}-ATPase in the presence of the organic solvent Me_2SO. A number of studies with different ATPases have shown that this solvent has a pleiotropic action on their catalytic cycles (for a review, see [6]). For the sarcoplasmic reticulum Ca^{2+}-ATPase, the addition of Me_2SO inhibits several partial reactions among them the forward steps of ATP hydrolysis. The cumulative effect favors phosphorylation by P_i and ATP-P_i exchange, affects the affinity of the enzyme for substrates [7] and uncouples the phosphorylation by ATP from Ca^{2+} binding to the transport sites [4,8]. For the Na^+/K^+-ATPase similar effects have been described [9–11]. In the erythrocyte Ca^{2+}-ATPase, [12] it was demonstrated that Me_2SO increases phosphorylation by P_i. In all of these cases, it has been proposed that Me_2SO exerts its effects on the intermediate species related to the E_2 conformation.

Activation of pNPP hydrolysis by Me_2SO has also been described for several of these enzymes. For the Na^+/K^+-ATPase, a small increase in affinity for pNPP with no significant change in V_{max} has been described [13]. For yeast plasma membrane H^+-ATPase, large increases in both affinity for substrate and V_{max} were observed [14]. Again, in all of these cases, it has been proposed that Me_2SO stabilizes the intermediate species related to the E_2 conformation.

However, for the erythrocyte Ca^{2+}-ATPase, Me_2SO shows an additional feature: it has been proposed that it can mimic calmodulin and (or) phospholipids because in the presence of this organic solvent, the enzyme exhibits a higher affinity for calcium and a higher V_{max} for ATP hydrolysis [15,16]. Using a delipidated and calmodulin-independent preparation, Lehotsky et al. [17] proposed that, in the presence of EGTA, Me_2SO can substitute for phospholipids in activating pNPP hydrolysis. Accordingly, binding of calmodulin to the regulatory domain and modulation of the enzyme by acidic phospholipids or by self-association have been reported to be mediated by hydrophobic interactions [15,18–21].

Here we show that the solvent increases the phosphatase activity of erythrocyte Ca^{2+}-ATPase by a particular mechanism which makes the activity to become independent of Ca^{2+} binding to the enzyme's high affinity transport sites. The data suggest that in the presence of Me_2SO the E_1 form, which is favored by the binding of calcium, is also able to hydrolyze pNPP. This is an unique and remarkable effect of the solvent on the plasma membrane Ca^{2+}-ATPase, which is similar, although exerted over E_1, to the increase of the reactivity of the E_2 conformer toward substrates described above for other P-type ATPases.

2. Materials and methods

pNPP, CaM, Na_2^+-ATP, Tris, EDTA, Hepes, phosphatidylcholine, polyoxyethylene 9 lauryl ether (polidocanol) and dithioerythritol were obtained from Sigma (USA). Me_2SO was from Merck. Calmodulin-Sepharose 4B resin was from Pharmacia. The enzymes for synthesis of radioactive ATP were from Boehringer Mannheim. $^{32}P_i$ was purchased from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; Calbiochem) was a generous gift from Dr. F. Guillain (Département de Biologie Cellulaire et Moleculaire, Centre d'Etudes de Saclay, Gif-sur-Ivette, France).

Calmodulin-depleted red cell ghost membranes

were prepared from fresh pig blood lysed isotonically in media containing 1 mM EGTA by freezing at −70°C, as previously described [44]. The protein concentration of ghost membranes was determined according to [22].

The plasma membrane Ca^{2+}-ATPase was purified from polidocanol solubilized ghost membranes by elution from a calmodulin-Sepharose affinity column [23] and stored in liquid nitrogen at a concentration of 100–200 μg/ml in a medium composed of 20 mM Hepes (pH 7.4), 0.6 M sucrose, 0.5 M KCl, 0.5% (v/v) polidocanol, 5 mM $MgCl_2$, 2 mM EDTA, 50 μM $CaCl_2$, 2 mM dithioerythritol and 0.25 mg/ml phosphatidylcholine (storage medium). The protein concentration was determined by the same method used for ghost membranes, after precipitation with deoxycholate and trichloroacetic acid [24].

For measurements of the Ca^{2+}-ATPase activity, approx. 3 μg/ml of enzyme was incubated for 60 min. at 37°C in 0.1 ml of a standard medium containing 50 mM Hepes (pH 7.2), 80 mM KCl, 10 mM $MgCl_2$ and 0.2 mM BAPTA, in the presence or absence of 13 mM pNPP. The Me_2SO and Ca^{2+} concentrations in each set of experiments are given in the figure legends. Free Ca^{2+} concentrations were calculated using the constants given by Tsien [25]. The reactions were initiated by addition of 1 mM [γ-^{32}P]ATP and stopped by trapping the nucleotide on charcoal suspended in 0.1 N HCl. The [^{32}P]P_i released was measured in the supernatant after centrifugation [26]. Largely different concentrations of enzyme (0.75–6.0 μg/ml) were used throughout this paper. As a control we observed that the enzyme remains fully sensitive to calmodulin. Thus, in the range of concentration we have used, increasing the concentration of protein does not cause oligomerization.

For measurements of pNPPase activity, release of pNP was detected spectrophotometrically at 425 nm. The standard medium was similar to that used for measurement of ATPase activity in the absence of ATP but presence of 13 mM pNPP, unless otherwise specified. The reaction was performed with 0.5 ml of this medium in a glass cuvette of 1.0 ml capacity. Small volumes of concentrated solutions of $CaCl_2$ were added sequentially to give the desired final concentrations, at each pH. pNPPase activity was totally insensitive to ouabain.

3. Results

In marked contrast with erythrocyte ghost preparations [27,28], the purified Ca^{2+}-ATPase displays a small but reproducible hydrolysis of pNPP in the absence of both ATP and Ca^{2+} ($[Ca^{2+}] < 0.01$ μM) as well as in the absence of CaM. pNPP hydrolysis is strongly stimulated (around 10-fold) by the presence of the solvent Me_2SO. This effect is not restricted to a single condition and is observed at both pH 7.2 and 8.0 (Fig. 1) as well as at KCl concentrations varying from 20 to 80 mM (not shown).

The pNPPase activity is inhibited by the raise in free Ca^{2+} concentration, the K_i for Ca^{2+} increasing with the addition of Me_2SO (Fig. 2A). This is better observed at pH 8.0 (Fig. 2B), where the affinity of the enzyme for Ca^{2+}, in the absence of the solvent, is greater [29,30]. Table 1 summarizes the different values of K_i and V_{max} calculated by fitting the curves

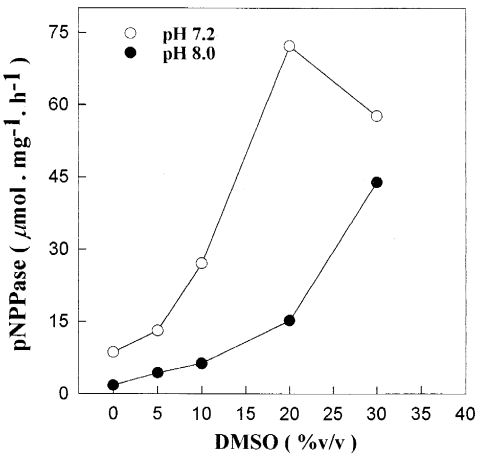

Fig. 1. Stimulation of pNPPase activity by Me_2SO. The standard medium contained 50 mM HEPES pH 7.2 (○) or 8.0 (●), 80 mM KCl, 10 mM $MgCl_2$, 0.2 mM BAPTA, $CaCl_2$ (free Ca^{2+}, 0.01 μM), 13 mM pNPP and Me_2SO concentrations shown on the abscissa, at 37°C. pNPPase activity was measured as described in Section 2. At pH 7.2 the reaction was performed with 2.25 μg/ml of purified ATPase in the absence of Me_2SO, and 0.75 μg/ml of protein in the presence of 5–30% Me_2SO. At pH 8.0 the reaction was performed with 6 μg/ml of purified ATPase in the absence of Me_2SO; 1.5 μg/ml of protein in the presence of 5–20% Me_2SO v/v, and 0.75 μg/ml of protein in the presence of 30% Me_2SO. ϵ_{425} for pNPP was, in aqueous media, 7500 $M^{-1}\cdot cm^{-1}$ at pH 7.2 and 11000 $M^{-1}\cdot cm^{-1}$ at pH 8.0. In the presence of Me_2SO, at concentrations 20% or higher, the ϵ_{425} determined was 12500 $M^{-1}\cdot cm^{-1}$ at pH 8.0, and 7400 $M^{-1}\cdot cm^{-1}$ at pH 7.2.

of Fig. 2. While the V_{max} increases, the K_i for inhibition of the pNPPase activity by Ca^{2+} varies with the solvent concentration from 2 μM to 2 mM at pH 8.0 and from 130 μM to 850 μM at pH 7.2.

The experiments above were performed in the absence of calmodulin. Under such conditions, only inhibition by Ca^{2+} is observed irrespective of the presence (triangles and squares) or absence (circles in Fig. 2A,B) of DMSO. When CaM is added in the absence of solvent, the dependence of pNPPase activity acquires a bell-shaped pattern and can be stimulated at Ca^{2+} concentrations below 10 μM (Fig. 3). This stimulation can be attributed to the binding of CaM to the enzyme at Ca^{2+} concentrations high enough to saturate CaM, but low enough to occupy the ATPase binding sites [27,28]. At higher Ca^{2+} concentrations, the binding of Ca^{2+} to the enzyme itself promotes the conversion of E_2 to E_1 and consequently inhibits pNPPase activity [27,31,32]. In the presence of calmodulin, addition of 30% Me_2SO increases pNPPase activity by 6-fold. In a solvent containing medium no activation by Ca^{2+} is detected, but inhibition is shifted to mM concentrations of Ca^{2+} (Fig. 3). The value of K_i obtained by fitting the curve is very close to that measured in the absence of CaM and presence of solvent in Fig. 2A. The differences in V_{max} are due to the different pool of preparations used for experiments depicted in Fig. 2 and Fig. 3. In fact, when the experiments are performed with the same preparation, the curves obtained in the presence and absence of CaM at 30% Me_2SO are indistinguishable and activation by CaM disappears (not shown). It is also noteworthy that activation in a Me_2SO containing medium surpasses by severalfold the maximal activation obtained in the presence of CaM, but absence of solvent.

Since inhibition by Ca^{2+} has been attributed to Ca^{2+} binding to the high affinity sites of the enzyme, the shift of the K_i observed in Fig. 2A,B could indicate that Me_2SO decreases the apparent affinity of the enzyme for Ca^{2+}. This, however, appears not to be the case. Fig. 4 shows that, at 10% Me_2SO, the $K_{0.5}$ for Ca^{2+} activation of ATP hydrolysis is 8.3 ± 3.6 μM. When the Me_2SO concentration is raised to 30% the maximal velocity is decreased (from 66.5 ± 6.5 to 22.4 ± 2.7 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$) and, concomitantly, the $K_{0.5}$ for Ca^{2+} activation is decreased to 2.6 ± 1.5 μM. These results are in agreement with previous reports of Benaim and de Meis [15,16] although with differences in cooperativity, taking into account the distinct experimental conditions. Thus, Me_2SO has an opposite effect, i.e., it increases the apparent affinity for Ca^{2+}. Furthermore, the values of $K_{0.5}$ for activation of ATPase activity by Ca^{2+} found at both Me_2SO concentrations are quite low compared to the K_i values for Ca^{2+} inhibition of pNPPase activity (compare with values shown in Table 1). This indicates that the inhibition of the pNPPase activity by Ca^{2+} in the presence of Me_2SO is not due to the binding of the cation to the high-affinity sites of the enzyme.

As a control, the ATPase activity was measured at different Ca^{2+} concentrations in the presence of 13 mM pNPP (not shown). In the presence of 10% or 30% Me_2SO, the Ca^{2+} concentration dependence was not significantly modified by pNPP, although

Table 1
K_i and Hill coefficients for Ca^{2+} dependence of inhibition of pNPP hydrolysis in the pNPPase activity at pH 7.2 and pH 8.0 in the presence of DMSO

	[DMSO] (v/v)	V_{max} (μmol pNPP · $mg^{-1} \cdot h^{-1}$)	K_i (μM)	n_H
pH 7.2	0%	8.67 ± 0.25	130 ± 22	0.74 ± 0.090
	10%	31.85 ± 0.85	262 ± 43	0.64 ± 0.080
	30%	83.05 ± 1.54	857 ± 114	0.38 ± 0.040
	30% + CaM	–	703 ± 111	0.41 ± 0.050
pH 8.0	0%	1.93 ± 0.12	2.7 ± 0.90	0.50 ± 0.06
	10%	4.83 ± 0.41	280.0 ± 180.0	0.24 ± 0.06
	30%	38.86 ± 0.78	2039.0 ± 0.77	0.73 ± 0.18

Conditions as described in Fig. 2. Data from 4–5 experiments.

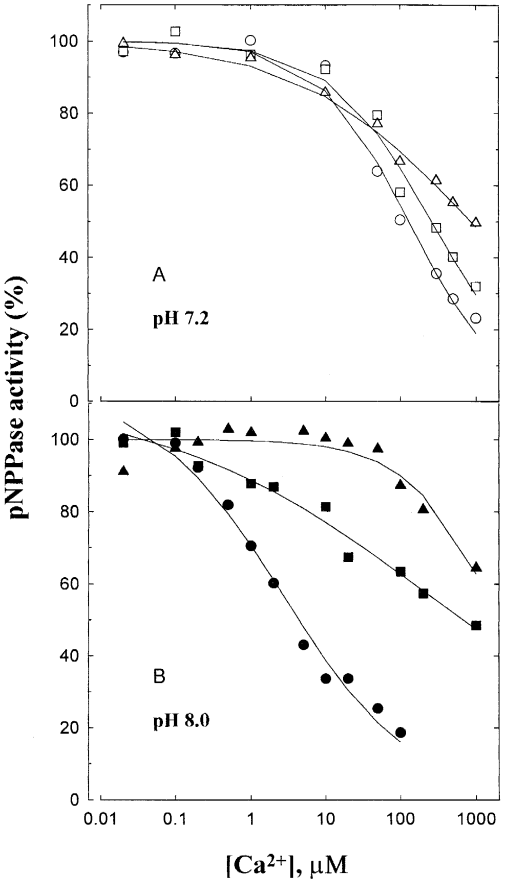

Fig. 2. Effects of Me_2SO on Ca^{2+} dependence of pNPP hydrolysis in the absence of CaM. pNNPase activity was measured in standard medium, with 13 mM pNPP and $CaCl_2$ added to give the free Ca^{2+} concn. indicated on the abscissa. (A) Reactions were performed at pH 7.2 with: (○) 2.5 μg/ml of purified ATPase in the absence of Me_2SO; (□, △) 0.75 μg/ml of protein with 10% and 30% Me_2SO v/v respectively. (B) Reactions were performed at pH 8.0, with (●) 6.0 μg/ml of protein in the absence of Me_2SO; (■) 1.5 μg/ml protein in the presence of 10% Me_2SO; (▲) 0.75 μg/ml protein in the presence of 30% Me_2SO. Curves were generated according to the equation, in which $v = \frac{V_{max} K_i^{n_H}}{K_i^{n_H} + [Ca^{2+}]^{n_H}}$ in which V_{max} is the maximal velocity in the absence of inhibitors, K_i is the Ca^{2+} concentration that inhibits activity by 50% and n_H is the Hill number. The parameters obtained by fitting are shown in Table 1. The points represent means of four experiments. Values of 100% of pNPPase activity correspond to the maximal velocities depicted in Table 1.

pNPP strongly inhibited the ATPase activity at high solvent concentrations. Thus, even in a medium containing both pNPP and Me_2SO, the equilibrium is dislocated towards E_1 at very low Ca^{2+} concentrations. This displacement, however, does not result in

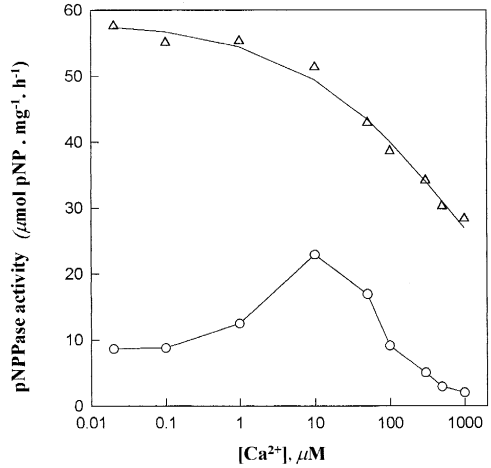

Fig. 3. Effects of Me_2SO on Ca^{2+} dependence of pNPP hydrolysis in the presence of CaM. The pNPPase activity was measured in standard medium at pH 7.2 and 13 mM pNPP, with 2.5 μg/ml of purified ATPase in the absence of Me_2SO (○), or 0.75 μg/ml of protein in the presence of 30% Me_2SO (△). The media contained 2.0 μg/ml calmodulin and $CaCl_2$ to give the free Ca^{2+} concentrations indicated on the abscissa. Curve shown for data at 30% Me_2SO was generated as in Fig 2. Values are means of five experiments.

abolishment of the pNPPase activity when the medium contains solvent.

In various P-type ATPases, it has been shown that Me_2SO and other organic solvents increase by sev-

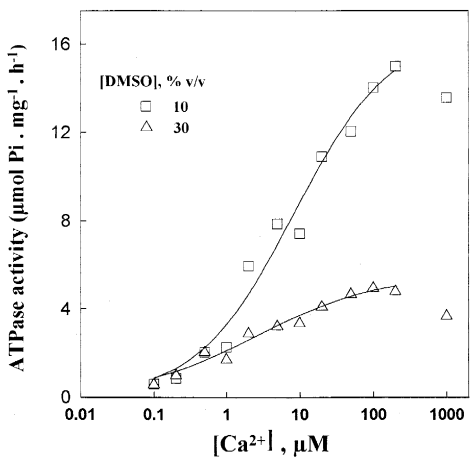

Fig. 4. Effects of Me_2SO on Ca^{2+} dependence of ATP hydrolysis. Reactions were performed at pH 7.2 in the absence of pNPP and either 10% Me_2SO (□) or 30% Me_2SO (△). The ATPase activity was measured in standard medium (see Section 2) during 60 min at 37°C. $CaCl_2$ was added to give the free Ca^{2+} concentrations indicated on the abscissa. Values are means of five experiments.

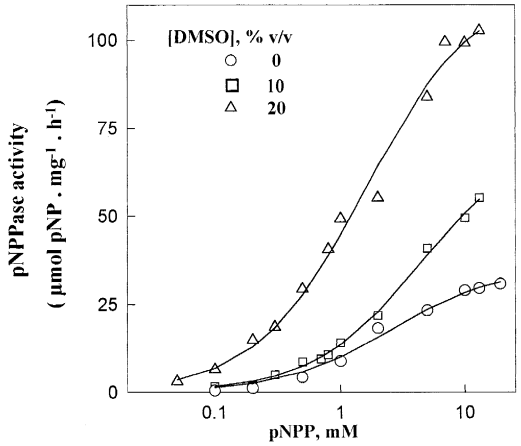

Fig. 5. pNPP dependence of pNPP hydrolysis at increasing Me_2SO concentrations. Enzyme was preincubated in the standard reaction medium at pH 7.2 and 0.01 μM free Ca^{2+}, with the indicated pNPP concentrations and either zero (○), 10% (□) or 20% (△) Me_2SO. pNPP hydrolysis was measured as described in Section 2.

eral orders of magnitude the apparent affinity of the enzymatic form E_2 towards a variety of ligands (for sarcoplasmic reticulum Ca^{2+}-ATPase: [4,7]; for Na^+/K^+-ATPase: [5,9,10]; for erythrocyte Ca^{2+}-ATPase: [12]). In erythrocyte Ca^{2+}-ATPase, however, the influence of Me_2SO on the K_m for pNPP is not so marked. When Me_2SO (20%) was added to the reaction medium, the K_m for pNPP decreased from 2.6 ± 0.4 to 1.6 ± 0.2 ($n = 3$) with a concomitant large increase in V_{max} (Fig. 5). This modest effect on apparent affinity indicates that the higher velocities observed in the presence of Me_2SO at low Ca^{2+} concentrations are rather due to an increase in the efficiency of enzyme catalysis.

4. Discussion

Several laboratories have shown that the Ca^{2+}-pumping ATPase of erythrocyte membranes displays a phosphatase activity (towards *p*-nitrophenylphosphate) that is activated by monovalent cations such as K^+ or Na^+ [33]. This activity is detectable on addition of either ATP or CaM and requires Mg^{2+}, provided that Ca^{2+} is present at the appropriate concentrations [3,34,35]. The phosphatase activity has been attributed to the low-affinity site for ATP, which is characteristic of the E_2 conformation [3].

In the absence of ATP but in the presence of calmodulin, Ca^{2+} at very low concentrations binds to calmodulin promoting the phosphatase activation, while at higher concentrations it inhibits the pNPPase activity of the plasma membrane Ca^{2+}-ATPase by displacing the equilibrium towards the E_1 conformer [3]. In the presence of ATP, the same concentration of Ca^{2+} activates the pNPPase activity, presumably by promoting the E_2 conformer during enzyme turnover [27]. Acidic phospholipids [36–38] and limited proteolysis [27], which removes the auto-inhibitory peptide, also activate plasma membrane pNPPase activity. In both cases, pNPP hydrolysis is impaired by Ca^{2+} binding to the high affinity sites, proposedly again by displacing the equilibrium toward E_1.

In this report, the organic solvent Me_2SO has been used in a study of the hydrolysis of pNPP by the plasma membrane Ca^{2+}-ATPase. The solvent has been reported both to favor a catalytically competent E_2 conformation of P-type ATPases [12] and to substitute for CaM and/or phospholipids as an activator of ATP hydrolysis by erythrocyte Ca^{2+}-ATPase [15,16]. Here we show that Me_2SO exerts complex and multiple effects on pNPP hydrolysis, among them it causes an increase in reactivity of the E_1 conformer towards pNPP, an effect that seems to be unique for the plasma membrane Ca^{2+}-ATPase.

In line with this proposal, Rossi et al. [27] reported that in aqueous medium, pNPP does not influence the equilibrium between the two conformers E_1 and E_2. They showed that both conformations can bind pNPP with similar affinities, but E_1 cannot proceed the catalytic steps as E_2 does. Their observations could explain why the organic solvent has a much more pronounced effect on V_{max} than on the K_m for pNPP (Fig. 5). We propose that Me_2SO alters the E_1 conformation, so that it acquires the ability to catalyse pNPP hydrolysis similar to that of E_2.

The molecular mechanism by which Me_2SO modifies the reactivity of substrates towards the E_2 form of P-type enzymes is controversial. It is apparent that Me_2SO promotes changes in protein structure. When Me_2SO is added to an aqueous protein solution, a gradual reversible destabilization of the secondary structure takes place [39]. At a concentration of 65%, Me_2SO induces significant changes in protein structure, with concomitant aggregation. Our experiments, however, were carried out at concentrations below

this apparently critical level. Using an iodoacetamide spin label, Coan [40] showed that 30% Me_2SO modifies the sarcoplasmic reticulum Ca^{2+}-ATPase in the same direction that Ca^{2+} does. With the yeast plasma membrane H^+-ATPase, on the other hand, addition of 20% (v/v) Me_2SO modified the pattern of trypsinization towards a conformation similar, but not identical to E_2 [14]. For the Na^+/K^+-ATPase, it has been proposed that Me_2SO promotes a rearrangement of the catalytic site of the dephosphorylated enzyme so that it attains a transition-state configuration similar to that of the E_2 phosphorylated intermediate [41]. Such a conformation would account for a simultaneous increase in enzyme affinity for effectors as dissimilar as P_i, $Mg^{2+}$and ouabain [41]. Although the exact conformation adopted by these enzymes at variable concentrations of the organic solvent are not clear, it is tempting to speculate that for the erythrocyte Ca^{2+}-ATPase a similar phenomenon occurs. A phosphorylated-like state of the active site could easily account for the increase in ability to catalyse pNPP hydrolysis.

Alternatively (or maybe concurrently with the effects above), the organic solvent may lower water activity in the active site, excluding water molecules, thus favoring catalysis. Regarding the E_2 conformation, a displacement of about 18 water molecules induced by Me_2SO has, indeed, been calculated for the phosphorylation by P_i of the Ca^{2+}-ATPase of sarcoplasmic reticulum [42] as well as a displacement of 5 molecules was calculated for the binding of F_4Al^- (as analogue of P_i) to the H^+-ATPase of yeast plasma membranes [43]. Regarding the E_1 conformation, there are no measurements concerning a possible role of water on catalysis. If the hypothesis of changes on water proved to be correct, the results shown in this work would imply that (at least concerning the PM Ca-ATPases) fluctuations of hydration of the catalytic site at the E_1 conformation may also increase the reactivity of the site with a consequent change in selectivity towards the substrates and may reveal the importance of water for the chemical reactions of the whole cycle.

Inhibition of the pNPPase activity by high concentrations of Ca^{2+} observed in the presence of Me_2SO (Fig. 2) may be due to the binding of the cation at the low-affinity sites of the enzyme [16,20]. So, when we measure pNPPase activity at increasing Me_2SO concentrations, the lack of inhibition by the occupancy of the high-affinity sites would gradually reveal the existence of the low-affinity ones (see Fig. 2). Since Mg^{2+} has been shown to activate pNPP hydrolysis [27], high concentrations of Ca^{2+} could also inhibit by competing with Mg^{2+}. At the saturating Mg^{2+} concentrations used in this paper, however, this is highly unlikely.

In conclusion, a complex picture emerges from the effects of Me_2SO on plasma membrane Ca^{2+}-ATPase. Some of the effects are similar, and some are distinct from those exerted on other P-type ATPases.

(A) In a medium containing 0.01 μM Ca^{2+}, Me_2SO increases the reactivity of E_2 towards pNPP (this paper) as well as towards P_i [12] as a substrate. Such an effect is in line with that observed for other P-type ATPases, and the possible causes were discussed above at paragraphs 5 and 6.

(B) Me_2SO increases the enzyme's affinity for Ca^{2+} and modulates the ATP hydrolysis. Such effects have already been described, and they are confirmed in this paper (although with some quantitative variation). They have been attributed to a 'calmodulin like' effect. Due to its nature, this is a regulatory effect specific for the plasma membrane Ca^{2+}-ATPase. It has been attributed elsewhere to removal of the inhibitory peptide, which could lead to a shift towards E_1.

(C) Me_2SO, on the other hand, activates pNPP hydrolysis to a much higher extent than calmodulin does; surprisingly, the E_1 conformation acquires the ability to hydrolyse pNPP. Such effects are clearly distinct from those exerted by any other activator in the enzyme and do not agree with a simple 'calmodulin-like' effect as previously proposed. It may be attributed either to an additional cummulative effect (besides the 'calmodulin like' effect), or to a shift towards a conformation that is insensitive to calmodulin. The reasons for the increase in the E_1 reactivity are not clear at this point, but it appears to be observed only with the plasma membrane Ca^{2+}-ATPase among the P-type ATPases, since, under similar conditions, the NPPase activity of Na^+/K^+-ATPase is totally inhibited by Na^+ [2,13]. In any case, it is clear that the effects of Me_2SO on this particular system go far beyond an ability to mimick activators and are much more complex than the effects of calmodulin.

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