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The promiscuous phosphomonoestearase activity of *Archaeoglobus fulgidus* CopA, a thermophilic Cu^+ transport ATPase

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ABSTRACT

Membrane transport P-type ATPases display two characteristic enzymatic activities: a principal ATPase activity provides the driving force for ion transport across biological membranes, whereas a promiscuous secondary activity catalyzes the hydrolysis of phosphate monoesters. This last activity is usually denoted as the phosphatase activity of P-ATPases. In the present study, we characterize the phosphatase activity of the Cu^+-transport ATPase from *Archaeglobus fulgidus* (*Af*-CopA) and compare it with the principal ATPase activity. Our results show that the phosphatase turnover number was 20 times higher than that corresponding to the ATPase activity, but it is compensated by a high value of K_m, producing a less efficient catalysis for pNPP. This secondary activity is enhanced by Mg^{2+} (essential activator) and phospholipids (non-essential activator), and inhibited by salts and Cu^+. Transition state analysis of the catalyzed and noncatalyzed hydrolysis of pNPP indicates that *Af*-CopA enhances the reaction rates by a factor of 10^5 ($\Delta\Delta G^{\ddagger} = 38$ kJ/mol) mainly by reducing the enthalpy of activation ($\Delta\Delta H^{\ddagger} = 30$ kJ/mol), whereas the entropy of activation is less negative on the enzyme than in solution. For the ATPase activity, the decrease in the enthalpic component of the barrier is higher ($\Delta\Delta H^{\ddagger} = 39$ kJ/mol) and the entropic component is small on both the enzyme and in solution. These results suggest that different mechanisms are involved in the transference of the phosphoryl group of p-nitrophenyl phosphate and ATP.

1. Introduction

P_{1B}-ATPases constitutes a group of evolutionarily related integral membrane proteins widely distributed in all the domains of life (Eukarya, Bacteria, and Archaea), which are involved in the active transport of heavy metals (Cu^+, Zn^{2+}, Cd^{2+}, etc) through cellular membranes [1,2]. Particular attention has been given to these proteins because mutations in the human Cu-ATPase genes, ATP7A and ATP7B, lead to Menkes syndrome and Wilson's disease, respectively [3,4]. *Archaeoglobus fulgidus* CopA (*Af*-CopA) is one of the best-studied members of this group. It can be heterologously expressed in *E. coli*, purified by affinity chromatography, and reconstituted in mixed micelles composed of soybean phospholipids and DDM. Following this procedure, a functional protein is obtained which displays characteristic properties of a thermophilic enzyme with maximal activity at 75 °C [5], high stability against thermal [6] and chemical [7] denaturation. This behavior seems to not require the unique features of archaeal lipids (ether linkages and isoprenoid chains). Thus, these micelar preparations have become the standard ones for structural and functional studies. The protein consists of a single polypeptide chain of 804 amino acid residues and has eight transmembrane helices, and two large cytosolic loops comprising the actuator and the catalytic domains, and two metal binding domains in the N- and C-terminus [2]. Atomic resolution structures were obtained for the actuator [8] and the catalytic domain in the free [9] and nucleotide bound forms [10] as well as for the C-terminal metal binding domain [11]. Recently, two crystal structures of a complete P_{1B} Cu^+-ATPase from *Legionella pneumophila* were resolved at 3.2 Å (PDB 3RFU) [12] and 2.8 Å (PDB 4BBJ) [13] showing a core structure shared with other P-type ATPases but with a distinctive transmembrane organization.

Ion transport by *Af*-CopA begins with the Cu^+ transfer from a partnering intracellular metalochaperone to the transmembrane metal binding sites (TM-MBSs) located on the transmembrane helices 6, 7, and 8 [14,15]. Translocation of the bound Cu^+ is coupled to the binding and hydrolysis of ATP following a classical Albers-Post E_1/E_2 model characteristic of all P-type ATPases [16,17]. Upon ATP binding to the E_1 conformation, the enzyme is phosphorylated at the invariant Asp residue in the conserved DKTG motif located at the catalytic domain, and Cu^+ is

Abbreviations: *Af*-CopA, Cu(I)-transport ATPase from *Archaeglobus fulgidus*; DDM, n-dodecyl β-D-maltoside; DTT, Dithiothreitol; HAD, Haloacid dehalogenase; MOPS, 3-(N-morpholino)propanesulfonic acid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; Pi, Inorganic phosphate; X_{PL}, Phospholipid mole fraction in the micellar phase; PMCA, Plasma membrane Ca^{2+}-ATPase; PNPP, p-nitrophenyl phosphate; PS, Phosphatidylserine; SERCA, Sarcoplasmic reticulum Ca^{2+}-ATPase; TM-MBSs, Transmembrane metal binding sites.

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transiently occluded within the transmembrane region giving the E_1P intermediary. A conformational change is then triggered, forming the E_2P intermediary, by opening the TM-MBs to the extracellular milieu. Finally, the ion is released and the enzyme is dephosphorylated to the E_2 conformation that shifts to the conformation E_1 in which the TM-MBs are open toward the cytoplasmic side and is able to initiate a new reaction cycle.

In addition to the physiologically relevant ATPase activity, some well-characterized P-type ATPases (e.g. the Na^+/K^+-ATPase [18], the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) [19], and the plasma membrane Ca^{2+}-ATPase (PMCA) [20]) are able to catalyze the hydrolysis of phosphate monoesters like acetyl-phosphate and p-nitrophenyl phosphate (pNPP) in the absence of the transported ion. This activity, usually denoted as the "phosphatase" activity of P-ATPases, was also found in heavy metal P1B-ATPases. We have reported pNPPase activity in a purified preparation of CopA from *Archaeglobus fulgidus* in the absence of Cu^+ [21]. In contrast, Hatori et al. showed that another thermophilic Cu^+-ATPase, CopA from *Thermotoga maritima*, exhibits pNPPase activity but only in the presence of Cu^+ [22].

Secondary catalytic activities have been described for many enzymes, and it is believed that it could be the rule more than the exception [23]. The term *enzyme promiscuity* was introduced for denoting these activities other than the activity for which an enzyme evolved and that are not part of the organism's physiology [24]. It is believed that contemporary enzymes have evolved from multifunctional generalist ancestral enzymes and that natural selection gave rise to their increased catalytic specificity and efficiency [23]. However, if a physiologically non-relevant activity is not detrimental to the organism it could persist through the evolution [25].

In the present study, we characterize the promiscuous phosphomonoesterase activity of CopA from *Archaeglobus fulgidus* using p-nitrophenyl phosphate as substrate, comparing it with the principal ATPase activity, and explore its regulation by different ligands.

2. Materials and methods

2.1. Reagents

All chemicals used in this work were of analytical grade and purchased mostly from Sigma-Aldrich Co. p-nitrophenyl phosphate (di Tris salt) was provided by Calbiochem. All the phospholipids used in this work were purchased from Avanti Polar Lipids and dodecyl maltoside (DDM) was provided by Anatrace.

2.2. Af-CopA expression and purification

A. fulgidus CopA, cloned into pBADTOPO/His vector, was expressed in *E. coli* Top10 cells and purified essentially as reported [5]. The purified protein migrates as a single band in Tris-tricine SDS-PAGE [26]. *Af*-CopA concentration was determined using a molar extinction coefficient of 39,700 M^{-1} cm^{-1} [7].

2.3. Phosphomonoesterase turnover rate of Af-CopA

The turnover rate for the pNPPase activity of *Af*-CopA was determined as the initial rate of release of p-nitrophenol from pNPP per mole of enzyme. The phosphatase reaction medium contained 0.15 μM purified *Af*-CopA, 150 μM soybean phospholipids, 200 μM DDM, 50 mM MOPS-Na (pH 6.1 at working temperature), 60 mM pNPP, 30 mM $MgCl_2$, and 2.5 mM DTT. The reaction mixture (final volume 250 μl) was incubated at selected temperatures for several time periods, returned to an ice-bath and mixed with 1 ml of 0.5 M NaOH. Hydrolysis of pNPP was estimated by measuring the absorption of p-nitrophenol at 410 nm using a molar extinction coefficient of 17,000 M^{-1} cm^{-1} [18]. Nonenzymatic hydrolysis of pNPP was determined in the same reaction medium without *Af*-CopA.

2.4. ATPase turnover rate of Af-CopA

ATPase turnover rate was determined as the initial rate of release of Pi from ATP per mole of enzyme. The ATPase reaction medium contained 0.15 μM purified *Af*-CopA, 150 μM soybean phospholipids, 200 μM DDM, 50 mM MOPS-Na (pH 6.1 at working temperature), 3 mM $MgCl_2$, 800 mM NaCl, 3 mM ATP, 0.1 mM $CuSO_4$, and 2.5 mM DTT. Released Pi was determined in accordance with Lanzetta et al [27]. Nonenzymatic hydrolysis of ATP was determined in the same reaction medium without *Af*-CopA.

2.5. Data analysis

At least three independent preparations of purified *Af*-CopA were evaluated for each experiment. The equations were fitted to the experimental data using a non-linear regression procedure implemented on an Excel spreadsheet [28]. The dependent variable was assumed to be homoscedastic (constant variance), and the independent variable was considered to have negligible error. The "equation of best-fit" was deemed that which gave the minimal Akaike index [29] and the least biased fit. Parameters were expressed as the mean ± standard error.

3. Results

3.1. The phosphomonoestearase activity of Af-CopA is more effective but less efficient than the principal ATPase activity

The first step in characterizing the phosphatase activity of *A. fulgidus* CopA was to determine the dependence of the reaction rate on the substrate concentration at 75 °C, i.e. the optimal temperature for the principal ATPase activity [5]. It can be observed in Fig. 1A that the dependence of the turnover rate on pNPP concentration is well described by a hyperbolic function reaching an asymptotic value (k_{cat}) of 5.9 ± 0.3 s^{-1} with a K_m value for pNPP of 9 ± 1 mM. The principal ATPase activity also show a hyperbolic dependence on the substrate concentration (Fig. 1B), being the best-fit parameter values $k_{cat} = 0.26 \pm 0.01$ s^{-1} and $K_m = 0.11 \pm 0.02$ mM.

Comparison of the ability of *Af*-CopA to catalyze the hydrolysis of pNPP respect to ATP requires the measurement of the spontaneous hydrolysis of both substrates in the absence of enzyme (k_{non}). These measurements were performed in the same reaction medium used for the measurements of the enzyme activities, and at the same temperatures. Results of these experiments are shown in the Supporting Information (Figure S1). It can be observed that the rate coefficients for the nonenzymatic hydrolysis of ATP at 75 °C ($6.4 \pm 0.3 \cdot 10^{-6}$ s^{-1}) is similar to that corresponding to pNPP ($1.20 \pm 0.04 \cdot 10^{-5}$ s^{-1}), an observation that can be rationalized taking into account that both substrates have highly activated leaving groups. Comparing catalyzed and noncatalyzed kinetic constants (Table 1), it can be observed that the rate enhancement produced by *Af*-CopA is one order of magnitude higher for the hydrolysis of pNPP than for ATP.

On the other hand, the specificity of *Af*-CopA for pNPP respect to ATP can be evaluated by comparing the ratio k_{cat}/K_m for both activities (Table 1). Remarkably, despite the higher rate enhancement observed for the phosphomonoestearase activity, the enzyme is more specific for ATP. The origin of this higher specificity for the principal substrate is probably due to the structural complementarity between the ATP molecule and the nucleotide binding domain of the enzyme [10].

Another important parameter for evaluating the power of an enzyme as a catalyst is the enzyme proficiency [30] defined as the ratio between the second-order rate constant for the enzyme-substrate encounter (k_{cat}/K_m) and the rate constant in the absence of enzyme

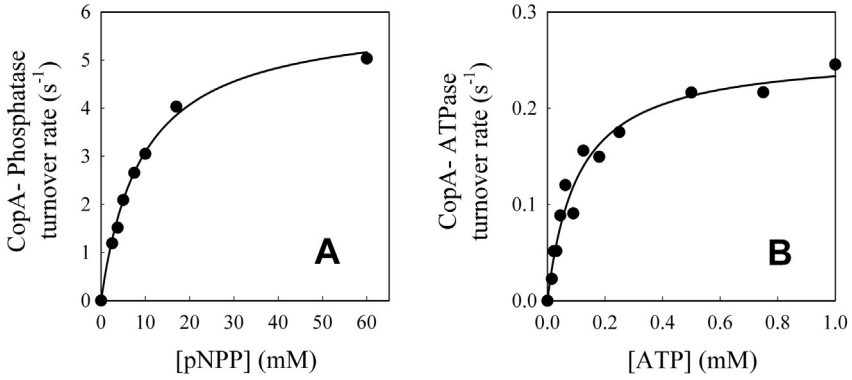

Fig. 1. The substrates curves of *Af*-CopA. Purified *Af*-CopA was incubated at 75 °C in the corresponding reaction medium with increasing concentrations of pNPP (A) or ATP (B) as indicated in the figure. The turnover rate was calculated for each condition and represented as a function of substrate concentration. Continuous lines are the graphical representation of a single hyperbolic function fitted to the experimental data with the parameter values indicated in the text.

(k_{non}). As we can observe, the catalytic proficiency of *Af*-CopA is only slightly higher toward its natural substrate.

Given the temperature dependence of the kinetic constants, we have repeated this analysis measuring the activities at 60 °C (Figure S3 and Table S1). It can be observed that the same conclusions can be drawn comparing the data at both temperatures.

3.2. Mg^{2+} is essential for the phosphomonoestearase activity of Af-CopA

One of the key events in the ATPase reaction cycle is the binding of Mg^{2+} to the ATP binding domain. It triggers the phosphorylation and induces the occlusion of the transported ions inside the transmembrane domain [10]. Fig. 2 shows that the phosphatase activity of *Af*-CopA increases with Mg^{2+} concentration. The best-fit model was a single hyperbolic function without intercept, indicating that Mg^{2+} is an essential activator of pNPP hydrolysis by *Af*-CopA. This activation was specific for Mg^{2+}, others divalent cations e.g. Mn^{2+}, Co^{2+}, Sr^{2+}, Ba^{2+} have no effect on the phosphatase activity.

3.3. Monovalent cations reduce the phosphomonoestearase activity of Af-CopA

It was previously described that Cu^{+} is an essential activator of the ATPase activity of *Af*-CopA [5]. Binding of Cu^{+} to the specific TM-MBS is required to initiate the ATPase reaction cycle of *Af*-CopA, and it is postulated that ion occlusion is coupled to the chemical step in which the γ-phosphate of ATP is transferred to Asp 424 [31]. To explore whether the phosphatase activity of *Af*-CopA is also regulated by Cu^{+}, the turnover rate was determined in the presence of different concentrations of this ion (Fig. 3). It can be observed that in contrast to that described for the ATPase activity, the phosphatase activity of *Af*-CopA decreases hyperbolically with increasing Cu^{+} concentration with $K_{0.5} = 0.06 \pm 0.01$ mM.

On the other hand, it was also described that the ATPase activity of *Af*-CopA is stimulated by high concentrations (400–500 mM) of NaCl [5]. To investigate if this characteristic is a general requirement of *Af*-CopA function, the effects of increasing NaCl concentrations were assayed on the phosphatase turnover rate (Fig. 4). It can be observed that activity was maximal in the absence of NaCl and decrease when increasing NaCl concentration, reaching a constant level at about 0.5 M. To determine if this effect is specific for NaCl, the turnover rate for the phosphatase activity of *Af*-CopA was determined in the presence of 800 mM NaCl, $NaNO_3$, or KCl. In all the cases, the measured activity was smaller than that measured in the control without added salts (Figure S4).

3.4. Phospholipids enhance the phosphomonoestearase activity of Af-CopA

It is recognized that phospholipids play an essential role in the biological function of integral membrane proteins [32]. To evaluate the regulation of the phosphatase activity of *Af*-CopA by phospholipids, the enzyme turnover rate of the purified protein reconstituted in detergent micelles was assessed by increasing the phospholipid mole fraction (X_{PL}) in the micellar phase (Fig. 5). As a first approach, we explored this regulation using soybean phospholipids, since all the available structural and functional information on this enzyme was obtained using this phospholipid mixture. Given the low critical micellar concentration of both amphiphiles, we consider that they are exclusively present in the micelles [33].

It can be observed that the addition of phospholipids produces a significant increase in *Af*-CopA phosphomonoestearase turnover rate reaching a maximum at mole fractions about 0.2. Similar experiments using some pure phospholipids showed that saturated fatty acid PC (16:0) was the more effective activator (Table 2). Changes in the polar head (PE, PS, or PI) or in the PC fatty acid composition appear to decrease the efficiency of *Af*-CopA for the hydrolysis of pNPP. Further studies are needed to explore these effects with archaeal lipids.

3.5. Temperature enhancement of the phosphomonoestearase activity of Af-CopA is lower than that of the principal ATPase activity

Fig. 6 shows temperature dependence of pNPPase turnover rate of purified *Af*-CopA in the range 30–100 °C. It can be observed that the enzyme turnover increases with temperature reaching a maximum between 70 and 80 °C. Further increase in temperature produces a fast inactivation of the enzyme by protein denaturation impeding the determination of the initial rate of pNPP hydrolysis (see Figure S2 in the supporting information). This dependence is similar to that previously reported for the ATPase activity of *Af*-CopA. However, the activation

Table 1
Rate enhancements and catalytic proficiencies of the principal (ATPase) and promiscuous (pNPPase) activities of *Archaeoglobus fulgidus* CopA at 75 °C.

Substrate	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{non} (s^{-1})	Rate enhancement k_{cat}/k_{non}	Catalytic proficiency (k_{cat}/K_m)/k_{non} (M^{-1})
pNPP	5.9	642	$1.20 \cdot 10^{-5}$	$5.0 \cdot 10^{5}$	$5.4 \cdot 10^{7}$
ATP	0.26	2437	$6.4 \cdot 10^{-6}$	$4.0 \cdot 10^{4}$	$3.8 \cdot 10^{8}$

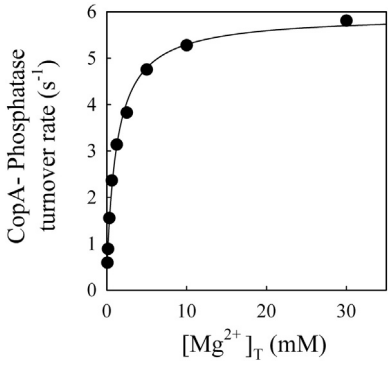

Fig. 2. Activation of *Af*-CopA catalyzed pNPP hydrolysis by Mg^{2+}. Purified *Af*-CopA was incubated at 75 °C in the phosphatase reaction medium with increasing concentrations of Mg^{2+} as indicated in the figure. The turnover rate was calculated for each condition and represented as a function of the total Mg^{2+} concentration. Continuous line is the graphical representation of a single hyperbolic function fitted to the experimental data. The best-fit parameter values were $k_{cat} = 5.8 \pm 0.1\ s^{-1}$ and $K_{05} = 1.0 \pm 0.1$ mM.

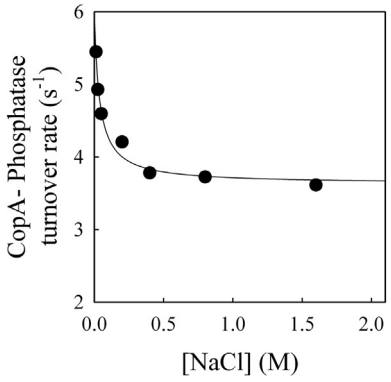

Fig. 4. Dependence of the pNPPase activity of *Af*-CopA on salt concentration. Purified *Af*-CopA was incubated at 75 °C in the phosphatase reaction medium with increasing concentrations of NaCl as indicated in the figure. The turnover rate was calculated for each condition and represented as a function of the concentration of salt.

energy calculated from the Arrhenius plot was 59 ± 3 kJ/mol, about a half of the value reported for the ATPase activity (103 ± 5 kJ/mol) [5], suggesting that different paths are involved in the hydrolysis of both substrates by the enzyme.

4. Discussion

4.1. Principal and promiscuous activities of Archaeglobus fulgidus CopA

The catalytic domain of the P-type ATPases shares a common fold within the haloacid dehalogenase (HAD) superfamily, with a set of conserved active site residues including the phosphorylatable aspartic acid residue [34]. HAD superfamily members are characterized by a broader specificity against different classes of substrates (sugars, nucleotides, organic acids, coenzymes, and small phosphodonors) [35,36]. Two characteristic enzymatic activities were described in P-type cation pumps: a principal ATPase activity and a secondary phosphomonoestearase activity usually denoted as the "phosphatase" activity of these enzymes. The principal activity provides the driving force for ion transport across cellular membranes. This activity appears quite specific for ATP, e.g., it was reported that no other nucleotide triphosphates can be hydrolyzed by the plasma membrane calcium pump [20], and very low activities were detected with UTP, CTP, GTP, and ITP in SERCA [37,38]. Despite this specificity for the hydrolysis of phosphoanhydride bonds, P-ATPases are able to catalyze the hydrolysis of phosphate monoesters like acetyl-phosphate and p-nitrophenyl phosphate [18]. Both activities appear to be independent, since modification of an specific Lys residue in the catalytic domain with fluorescein isothiocyanate abolish the ATPase, but not the phosphomonoestearase activity in SERCA [39] and PMCA [40].

Secondary activities resulting from the fortuitous use of active site features to catalyze an alternative reaction have been extensively characterized for many soluble enzymes [23–25]. These promiscuous activities are not detrimental for efficient catalysis of the primary reaction [41], and it was postulated that they provide the raw material for the evolution of new enzymes [23]. Membrane-associated enzymes constitute an important group of proteins characterized by being folded in a heterogeneous water-lipid environment [42]. Despite the essential cell functions in which they are involved, the relationship between their principal and secondary catalytic activities is far less characterized than in soluble ones.

It is accepted that catalysis of the secondary reaction is generally less efficient than on the primary function, with only few exceptions [25,43]. Comparisons like this are rare for membrane-related enzymes, and thus a clear trend cannot be drawn. For some P-type ATPases, the reported ATPase activities are higher in magnitude than the corresponding phosphatase activities [19,44], whereas in others, both activities are in the same order of magnitude [20,45]. Results presented in this work showed that *Achaeglobus fulgidus* CopA phosphatase turnover number was 20 times higher than that corresponding to the ATPase activity. This result suggests that the promiscuous phosphate monoester

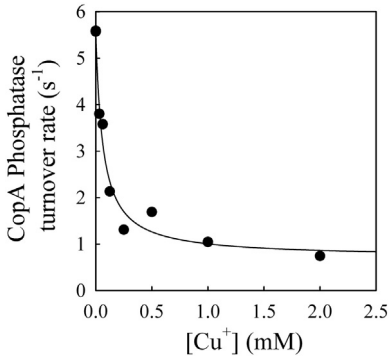

Fig. 3. Inhibition of *Af*-CopA catalyzed pNPP hydrolysis by Cu^{+}. Purified *Af*-CopA was incubated at 75 °C in the phosphatase reaction medium with increasing concentrations of $CuSO_4$ in the presence of a reducing agent. The turnover rate was calculated for each condition and represented as a function of the concentration of Cu^{+}. The best fit to the experimental data was achieved using a decreasing hyperbolic function. Continuous line is the graphical representation of this function with the following best-fit parameter values: $(A_o - A_\infty) = 4.8 \pm 0.7\ s^{-1}$; $K_{0.5} = 0.06 \pm 0.01$ mM.

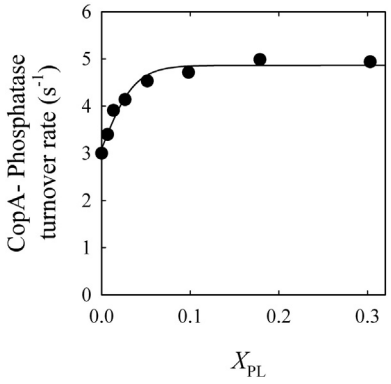

Fig. 5. Phospholipid modulation of *Af*-CopA pNPPase activity. Purified *Af*-CopA was incubated at 75 °C in the phosphatase reaction medium with increasing concentrations of soybean phospholipids as indicated in the figure. The turnover rate was calculated for each condition and represented as a function of phospholipid mole fraction in the micelar phase. Continuous line is the graphical representation of the minimal model described by Dodes Traian et al. [32] to the experimental data.

Table 2
Activation of *Af*-CopA pNPPase by different phospholipids.

Phospholipid	*Af*-CopA phosphatase turnover rate at $X_{PL} = 0.2$ (s^{-1})
PC (16:0)	8.2
PC (14:0)	7.6
PC (18:1)	4.9
PE (16:0)	5.5
PS (16:0)	5.1
Soybean phospholipids	5.9

PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine.

substrate would be better positioned than ATP relative to the catalytic machinery of the enzyme [24]. Conversely, the structural complementarity between the nucleotide binding domain of *Af*-CopA and ATP results in a smaller K_m value for the natural substrate. These features make *Af*-CopA an interesting system to explore the relationship between principal and promiscuous activities.

It is well established that turnover numbers provide a measure of the maximal capacity of an enzyme to catalyze the transformation of substrate into product. However, enzymes usually work in the presence of sub-saturating substrate concentrations under physiological conditions, thus allowing the reaction rate to respond to changes in substrate concentration during cell function. In these conditions, the reaction rate is governed by the second-order constant for the enzyme-substrate encounter (k_{cat}/K_m), a quantity denoted as the catalytic efficiency of the enzyme. It was proposed that enzyme evolution proceed via optimizing the catalytic efficiency [46] allowing the catalyzed reaction to occur on biologically relevant timescales [43]. Promiscuous substrates usually bind weakly to the enzyme resulting in high K_m values [24]. This is certainly the case for *Af*-CopA with a K_m for pNPP in the order of 10 mM. This high value of K_m compensates the high turnover rate, producing a less efficient catalysis for pNPP. In addition, the low values of k_{cat}/K_m for both activities—compare the values in Table 1 with $10^9\ M^{-1}\ s^{-1}$ corresponding to the so-called perfect enzymes [47]—indicate that the capture of the substrate is far to be diffusion limited.

The catalytic effectiveness of a given enzyme can be expressed in terms of the factor by which it enhances the rate of a reaction [30]. Comparisons of the rates of the enzyme-catalyzed reactions with the noncatalyzed background reaction gives an indication of the strength of interactions in the enzyme active site [30]. For this comparison, uncatalyzed and catalyzed reactions have to be measured in the same reaction medium and at the same temperature. Being *Af*-CopA a thermophilic enzyme with maximum activity at 75 °C, direct measurement of the nonenzymatic substrate hydrolysis is possible, avoiding the large extrapolation usually required for doing this comparison in mesophilic enzymes [30,48].

Our results show that the first-order kinetic coefficients for the noncatalyzed hydrolysis of pNPP and ATP are quite similar. Both substrates have highly activated leaving groups with pK_a values of 7.14 for p-nitrophenol [49] and 6.4 for ADP [50]. Despite these similarities, the temperature dependence of the rate coefficients is different for the noncatalyzed hydrolysis of ATP and pNPP, giving different enthalpic and entropic contributions to the free energy barrier (see the discussion in Section 4.3).

In this work, we show that the rate enhancement (k_{cat}/k_{non}) is one order of magnitude higher for the phosphomonoestearase activity of *Af*-CopA (Table 1). Nevertheless, the rate enhancement values obtained for both *Af*-CopA activities are low when compared with the previously reported values for soluble phosphate monoester hydrolases at 25 °C [36,51]. These low values result from the large differences in the rates of the noncatalyzed reactions rather than the catalyzed reactions, as was previously reported for many soluble enzymes.

Enzyme effectiveness as a catalyst can be also expressed as the ratio between the catalytic efficiency (k_{cat}/K_m) and the first-order rate constant for the uncatalyzed reaction k_{non} under the same conditions [30]. This ratio is called "catalytic proficiency" and gives a measure of the enzyme ability to reduce the activation barrier for the reaction of the substrate free in solution [30]. The catalytic proficiency of *Af*-CopA to hydrolyze ATP and pNPP are both similar and low when comparing with mesophilic phosphohydrolases [51]. Again, these differences arise from differences in the rates of the uncatalyzed reactions rather than the catalyzed reactions. The inverse of the enzyme proficiency has been shown to provide a lower limit for the association constant of the enzyme with the altered substrate in the transition state [52]. For *Af*-CopA, these constants are in the nanomolar range indicating a weak interaction in the transition states.

Low proficiencies are common in thermophilic soluble enzymes, indicating that enzymes working in warm environments do not need to enhance to much the rate of the reaction they catalyze [47]. As was mentioned, P-ATPases use the energy of ATP hydrolysis to actively transport ions across biological membranes. A high proficiency of these enzymes could result in transporting ions at a higher rate than required for maintaining cellular homeostasis. If this was the case, the enzyme could uncouple the enzyme activity to the transport. However, it was demonstrated that uncoupling ATP hydrolysis and transport results in heat production and normally occurs in situations where the cell needs to generate heat to maintain its temperature [53]. Certainly, this is not the case for a thermophilic organism living in its natural environment. In this way, our results allow to extend the hypothesis drawn by Wolfenden [47] postulating that in a warm environment, like that of the primordial biology, some metal-interacting hydrophobic proteins even with a modest enzyme activity would be enough to generate ionic gradients across cell membranes.

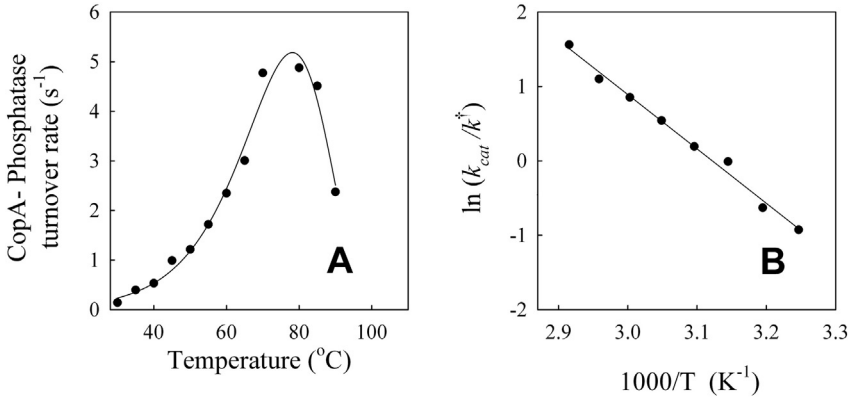

Fig. 6. Dependence of *Af*-CopA pNPPase activity on temperature. A: Purified *Af*-CopA was incubated in the phosphatase reaction medium at the temperatures indicated in the figure. The turnover rate was calculated for each condition and represented as a function of temperature. Continuous line is a guide for the eye. B: The Arrhenius plot is represented for the increasing arm of plot A. The solid line is the graphical representation of the Arrhenius equation fitted to the experimental data with the activation energy indicated in the text.

4.2. Regulation of the phosphatase activity of Archaeglobus fulgidus CopA

In this work, we demonstrate that Mg^{2+} is an essential activator for the phosphate monoesters hydrolase activity of *Af*-CopA, as was also reported for the principal ATPase activity [5]. We find that the enhancement of the pNPPase activity of *Af*-CopA by Mg^{2+} cannot be produced by other divalent cations. It was proposed that divalent metal ions can play several catalytic functions in phosphoryl transfer enzymes including a) activation of substrate by polarization of the phosphoryl group, b) activation of the nucleophile (water or an active site residue) by lowering the pKa of OH groups, c) offsetting negative charge on the oxygen of the leaving group [54]. For the ATPase activity of *Af*-CopA, Tsua and Toyoshima propose a possible scenario where Mg^{2+} dissociates from ATP during the catalytic cycle and binds to Asp 618 in the P domain, a coordination residue full conserved in all P-ATPases (Fig. 7) [10]. Thermal movements of the P domain would allow a change in ATP conformation with Mg^{2+} bridging the γ-phosphate, Asp 424, and Asp 618 in a similar way as was observed for SERCA [55]. In this form, the γ-phosphate of ATP is fixed by hydrogen bonds allowing its approximation to Asp 424 with the correct geometry. Then Mg^{2+} promotes charge rearrangements on the γ-phosphate and the carboxyl group of Asp 424 facilitating the formation of a covalent bond [10]. Phosphorylation of P-ATPases by pNPP has been previously reported [56], so that it is possible to postulate —as a first approximation—a similar role for Mg^{2+} in the phosphoryl transfer step. Furthermore, computational studies from Kamerlin et al. demonstrate that requirement of Mg^{2+} for phosphate ester hydrolysis may be a characteristic of an associative mechanism via a pentavalent phosphorus intermediate [57], in this case including coordination with the p-nitrophenyl leaving group and the Asp 424 nucleophile.

The principal ATPase activity of *Af*-CopA is also enhanced by Cu^{+} and Na^{+} [5]. Conversely, our results show that the promiscuous phosphatase activity is inhibited by these monovalent cations. The effect of NaCl appears to be a generalized nonspecific effect of salts. It is known that some charges at the surface of proteins are shielded at high salt concentration. Our results show that charge shielding produces opposing effects on both enzymatic activities, suggesting that some charged residues would favor the phosphatase activity whereas ATP hydrolysis would be favored by the absence of these charges. This observation suggests that different mechanisms are involved in the transfer of the phosphoryl group of pNPP and ATP. On the other hand, our experiments demonstrated that Cu^{+} is not necessary for pNPP hydrolysis thus suggesting that Cu^{+} is not transported during pNPP hydrolysis cycle. These results are in accordance with a previous mutagenesis study where replacing the residues involved in binding of Cu^{+} produce the lack of ATPase activity but does not abolish the phosphatase activity

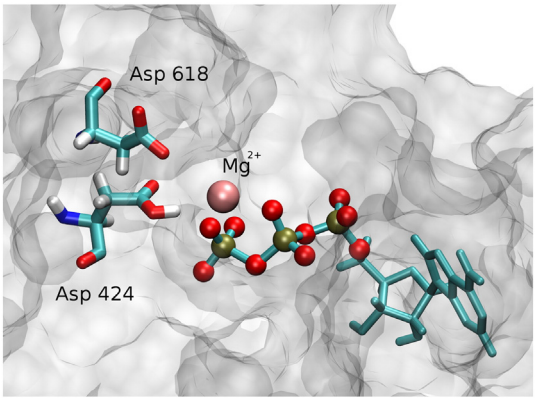

Fig. 7. The catalytic site *Af*-CopA. Detailed view of the interactions of ATP with the contours of the binding pocket in the ATPBD. ATP and interacting residues are shown in a ball and stick representation with atoms colored by atom-type: C, cyan; O, red; N, blue, H, white. Mg^{2+} is represented by a pink sphere. The picture is rendered with VMD 1.9.1 [58].

[59]. We also observe that high Cu^{+} concentration inhibits the phosphatase activity of *Af*-CopA. This effect cannot be identified as a salt effect because it occurs at lower concentrations, thus a specific effect of Cu^{+} should be operating. A possible explanation is that the conformation of *Af*-CopA induced by Cu^{+} (i.e. E_1–Cu^{+}), is less or not able to catalyze the hydrolysis of phosphate monoesters. A similar hypothesis was drawn for SERCA for which the phosphatase activity was postulated to be specific of the E_2 conformation [39].

Phospholipids also produce an increase in the phosphatase activity of *Af*-CopA, but in this case, the activation is not essential since a significant activity (near half of the maximal activity) was measured on the delipidated preparation of this enzyme. This is an important difference respect the principal activity which is very small—about 10 fold lower—in the absence of added phospholipids (Cattoni DI, PhD Thesis, Universidad de Buenos Aires). The modulation by phospholipids is highly cooperative (being the cooperativity factor [32] $c = 54 \pm 8$), reaching the maximal activity when phospholipids constitute only the 10% of the amphiphiles in the reconstitution medium. It was demonstrated that preferential interaction of phospholipids with the transmembrane helices induces conformational changes in the transmembrane domain of membrane proteins [32]. This change would be translated to all the conformations of the ATP binding domain along the catalytic cycle giving a more active enzyme. The relatively low enhancement by phospholipids suggests that the promiscuous phosphatase activity is mainly a property of the cytoplasmic catalytic domain of *Af*-CopA.

4.3. Transition state analysis of the principal and promiscuous activities of Archaeglobus fulgidus CopA

It is well established that the large catalytic efficiency of an enzyme can be accomplished by interacting differentially with the substrate in the ground and activated states. However, the mechanism through which enzymes reduce the energetic barrier is still not clear [46]. To explore the origins of the catalysis of pNPP hydrolysis by *Af*-CopA, we analyze the variation in the kinetic coefficients k_{cat} and k_{non} with the temperature in terms of a Kramers-type model [60,61] (Table 3):

$$k = \nu \frac{\eta_o}{\eta_T} e^{-\frac{\Delta G^{\ddagger}}{RT}} = \nu \frac{\eta_o}{\eta_T} e^{-\frac{\Delta H^{\ddagger}}{RT}} e^{\frac{\Delta S^{\ddagger}}{R}}$$

where ν represents the k value at the reference temperature for a barrier-less process [62], η is the viscosity of the medium at a reference temperature (η_0) and at the incubation temperature (η_T), and $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ are the activation free energy, enthalpy, and entropy, respectively.

In this analysis, the reference temperature was selected as 75 °C and the initial parameter values were taken as those calculated using the classical transition state theory. It is worth noting that the viscosity effect included in the Kramers model is critical in this work because of the wide temperature range used in the experiments.

It can be observed that the free energy barrier for the noncatalyzed hydrolysis of both substrates is similar, with a dominant enthalpic term which is significantly higher for the hydrolysis of ATP. Conversely, the activation entropy is close to zero for the ATP hydrolysis but negative for the hydrolysis of pNPP. Near-zero activation entropies have been described in solvolysis reactions not involving a molecule of solvent in the transition state [63]. On the other hand, negative activation entropies values have been previously reported for the noncatalyzed hydrolysis of phosphate monoesters and indicate that the degrees of freedom of the phosphomonoester and water are decreased in the transition state respect to the initial nonreacting state. These differences suggest that, beyond the abovementioned similarities, both substrates undergo nonenzymatic hydrolysis through different mechanisms.

Comparing $\Delta H^{\ddagger}$ values for k_{cat} with those corresponding to the nonenzymatic reactions, it is evident that *Af*-CopA act on both substrates by

Table 3
Thermodynamic activation parameters at 75 °C for the noncatalyzed, the principal (ATPase) and promiscuous (pNPPase) activities of *Archaeoglobus fulgidus* CopA.

	ATP non	ATP *Af*-CopA	pNPP non	pNPP *Af*-CopA
k (s^{-1})	$(6.4 \pm 0.3) \cdot 10^{-6}$	0.26 ± 0.01	$(1.20 \pm 0.04) \cdot 10^{-5}$	5.9 ± 0.3
$\Delta G^{\ddagger}$ (kJ/mol)	120.4 ± 0.2	89.6 ± 0.1	118.4 ± 0.1	80.5 ± 0.1
$\Delta H^{\ddagger}$ (kJ/mol)	126 ± 9	87 ± 3	79 ± 3	49 ± 4
$T\Delta S^{\ddagger}$ (kJ/mol)	6 ± 9	1 ± 3	-40 ± 3	-32 ± 3
$\Delta\Delta G^{\ddagger}$ (kJ/mol)	–	−30.8	–	−37.9
$\Delta\Delta H^{\ddagger}$ (kJ/mol)	–	−39	–	−30
$T\Delta\Delta S^{\ddagger}$ (kJ/mol)	–	−5	–	8

lowering the enthalpy of activation. Full comparison between the catalyzed and noncatalyzed hydrolysis of pNPP indicates that *Af*-CopA enhances the reaction rates by a factor of 10^5 ($\Delta\Delta G^{\ddagger} = 38$ kJ/mol) mainly by reducing the enthalpy of activation ($\Delta\Delta H^{\ddagger} = 30$ kJ/mol). For the ATPase activity, the decrease in the enthalpic component of the barrier is higher ($\Delta\Delta H^{\ddagger} = 39$ kJ/mol). Activation entropies are only slightly different on the enzyme than in solution for both activities. The enthalpic basis of rate enhancements have been interpreted in terms of new electrostatic and hydrogen bonds as responsible for binding the altered substrate in the transition state [64]. These mechanisms are commonly found in soluble enzymes [47]. Our results suggest that this evolutive feature also holds for membrane related enzymes.

5. Concluding remarks

In this work, we characterize the phosphatase activity of CopA from *Archaeglobus fulgidus*. This activity seems to be a property of the catalytic domain E_2 conformation of *Af*-CopA that shows distinctive features compared with the principal ATPase activity. Our results show that *Af*-CopA is more efficient for capturing ATP, but the promiscuous phosphate monoester substrate would be better placed at the catalytic site than ATP. Requirement of Mg^{2+} suggest an associative mechanism for phosphoryl transfer and the salt inhibition allow to propose that charged residues play an important role for the phosphatase activity whereas ATP hydrolysis would be favored by the absence of charges. Despite these differences in the mechanism, catalysis is driven in both cases by an enthalpy-lowering mechanism, and this effect is more prominent for the principal activity allowing a better response against changes in temperature.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

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