Involvement of an arginyl residue in the nucleotide-binding site of Ca^{2+} -ATPase from sarcoplasmic reticulum as seen by reaction with phenylglyoxal

Senena CORBALÁN-GARCÍA, José A. TERUEL* and Juan C. GÓMEZ-FERNÁNDEZ

Departamento de Bioquímica y Biología Molecular A, Edificio de Veterinaria, Universidad de Murcia, Apartado de Correos 4021, E-30080 Murcia, Spain

1. Chemical modification of the Ca^{2+} -ATPase with phenylglyoxal, as a modifier of arginine residues, leads to an almost total loss of the ATPase activity. The presence of nucleotides in the reaction medium protects against the binding of 18 nmol of phenylglyoxal/mg of protein and this reduction in the binding of phenylglyoxal is accompanied by a substantial retention of ATPase activity. The incorporation of phenylglyoxal to the protein alters neither calcium binding nor phosphorylation from inorganic phosphate. Nevertheless the binding of nucleotides is dramatically inhibited and, consequently, so is phosphorylation from ATP. Fluorescein 5'-isothiocyanate labelling of the phenylglyoxal-modified ATPase is not affected but, on the other hand, phenylglyoxal is not able to modify the fluorescein 5'-isothiocyanate-prelabelled ATPase. The way in which ATPase inhibition depends on the presence of phenylglyoxal indicates

that this process occurs in a pseudo-first-order reaction. However, the dependence of the apparent first-order rate constant on phenylglyoxal concentration appears to be more complex and an inhibition mechanism of two steps, with phenylglyoxal binding, has to be taken into account. 2. We have found that phenylglyoxal labels both A and B tryptic fragments, but only B fragment labelling is prevented by ATP. The sequencing of peptides from mild acid hydrolysis of phenylglyoxal-labelled ATPase shows that phenylglyoxal is located in the Ala⁵⁰⁶–Gly⁵⁹⁵ peptide that is a part of the B fragment. 3. We conclude that phenylglyoxal inactivates the calcium pump in a two-step mechanism in which the second step is irreversible. Phenylglyoxal labels an arginyl residue in the Ala⁵⁰⁶–Gly⁵⁹⁵ peptide that can be protected by the binding of ATP to its site.

INTRODUCTION

The Ca^{2+} -ATPase of sarcoplasmic reticulum (SR) from skeletal muscle is a single polypeptide chain of 110 kDa, of which the amino acid sequence has been determined from its cDNA [1]. It catalyses the hydrolysis of one molecule of ATP coupled to the translocation of two calcium ions, the formation of a phosphoenzyme intermediate being an essential step in the catalysis [2].

Trypsin specifically cleaves the Ca^{2+} -ATPase at two sites. First, it cleaves at Arg⁵⁰⁵ producing A (55 kDa) and B (54 kDa) peptides, and subsequently the A fragment is cleaved at Arg¹⁹⁸ to yield A2 (22 kDa) and A1 (33 kDa) subfragments. The cytoplasmic region contains the nucleotide-binding site (B fragment) as well as the phosphorylation domain (A1 subfragment). Several amino acids have been identified to as being involved in the formation of these domains. Thus, Asp³⁵¹ becomes phosphorylated by nucleotides and other energy-rich substrates, i.e. acetyl phosphate, *p*-nitrophenyl phosphate, or even by inorganic phosphate in the reversal of the catalytic cycle [3–5]. The nucleotide site has been addressed by different approaches: chemical modification of some residues [6–15], antibodies against different fragments [16,17], and site-directed mutagenesis [18,19]. All these experiments agree with participation of fragments around amino acid residues 500, 600, 670 (B fragment) and 350 (A1 subfragment) in the nucleotide-binding site.

Phenylglyoxal (PGO) modification has been used in many proteins as a specific reagent of arginine residues, such as in Ca^{2+} -ATPase from cardiac muscle [20], amino acid oxidase [21], phosphomannose isomerase [22], or aldose reductase [23]. In this study we have characterized the chemical modification of the Ca^{2+} -ATPase by PGO to specifically inhibit nucleotide binding, and in this way to gain further insight into the ATPase structure.

MATERIALS AND METHODS

Materials

PGO, fluorescein 5'-isothiocyanate (FITC), nucleotides, fluorescamine and the molecular-mass markers for SDS/polyacrylamide gel electrophoresis were from Sigma. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate and NADH were from Boehringer Mannheim. The radiochemicals, [³²P]P_i, [³²P]ATP, [⁴⁵Ca]CaCl₂, [¹⁴C]PGO and [³H]glucose, were purchased from New England Nuclear. The calcium ionophore, A23187, was obtained from Calbiochem. The chemicals used in the gel electrophoresis were from Bio-Rad. All other chemicals were obtained from Merck. SR vesicles were prepared by the method of Eletr and Inesi [24].

Chemical modifications

PGO modification was carried out as follows: 2 mg/ml SR was incubated for different periods of time at 25 °C in a medium containing 20 mM Mops/triethylamine, pH 8.0, 80 mM KCl, 1 mM EGTA, and a variable concentration of PGO. Then the vesicles were centrifuged at 100 000 *g* for 30 min at 4 °C and washed with 20 mM Mops, pH 6.8, 80 mM KCl [25].

Native and PGO-modified SR was labelled with FITC as described by Teruel and Inesi [10].

ATPase activity

ATPase activity was measured at 25 °C by a coupled enzyme system consisting of 0.02 mg/ml SR vesicles, 20 mM Mops,

Abbreviations used: FITC, fluorescein 5'-isothiocyanate; PGO, phenylglyoxal; SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid; TNP, 2'-(3')-O-(2,4,6-trinitrocyclohexadienylidene); PVDF, poly(vinylidene difluoride).

* To whom correspondence should be addressed.

pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂, 10 μ M A23187, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.24 mM NADH, 5 units/ml pyruvate kinase and 5 units/ml lactate dehydrogenase. Absorbance decay was measured at 340 nm, assuming an $\epsilon_{340}^{\text{NADH}} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

PGO binding

[¹⁴C]PGO binding was performed by a filtration method: SR vesicles were incubated in the same conditions as described above for PGO modification, but in the presence and absence of 0.5 mM ATP. Aliquots (80 μ g) were diluted in 1 ml of 20 mM Mops, pH 6.8, 80 mM KCl, 1 mM EGTA at 4 °C. Afterwards 0.9 ml of this solution was layered on a Millipore filter (HAWP, 0.45 μ m pore size) and washed with 15 ml of the dilution buffer.

Nucleotide binding

[γ -³²P]ATP binding was measured by a filtration method: a Millipore HAWP filter was first loaded with 0.4 mg of protein incubated at 25 °C in a medium containing 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂ and 1 mM EGTA. The filter was then perfused with 3 ml of the same medium containing different ATP concentrations (1–200 μ M) and 1 mM [³H]glucose. Glucose was added to determine the amount of substrate that is trapped in the filter, but not bound to the Ca²⁺-ATPase.

TNP [2'(3')-O-(2,4,6-trinitrocyclohexadienylidene)]-nucleotide binding was performed by a spectrophotometric method as described by Watanabe and Inesi [26].

Calcium binding

⁴⁵Ca²⁺ binding to modified and non-modified SR vesicles with PGO was measured at 25 °C by incubating 1.5 mg of vesicles/ml in a buffer containing 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM [³H]glucose, 100 μ M ⁴⁵CaCl₂ and various EGTA concentrations to reach the desired value of pCa. After an incubation time of 15 min, a portion (0.2 ml) was filtered through a Millipore filter (HAWP, 0.45 μ m pore size), and 0.05 ml was used to determine the ⁴⁵Ca²⁺ trapped in the filter but not bound to the protein [27].

SR phosphorylation

Phosphorylation of native SR and PGO-modified SR with inorganic phosphate was achieved by incubating SR vesicles (1 mg/ml) for 5 min at 25 °C in a medium containing 20 mM Mes, pH 6.0, 5 mM MgCl₂, 1 mM EGTA and different concentrations of [³²P]P_i from 0 to 5 mM (for ATP synthesis experiments 75 μ M A23187 was also included). Phosphorylation with ATP was achieved by incubating SR vesicles (1 mg/ml) for 30 s at 25 °C in a medium containing 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂ and 1 mM [γ -³²P]ATP. The phosphorylation reaction was quenched by addition of 0.25 M perchloric acid and 4 mM P_i. The quenched samples were cooled down on ice, washed and centrifuged repeatedly. The final pellets were dissolved to determine ³²P and protein concentrations. [γ -³²P]ATP synthesis was obtained by diluting the phosphorylated enzyme from P_i in order to yield a final concentration of 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 20 mM CaCl₂ and 2 mM ADP. [γ -³²P]ATP synthesis was determined by measuring the radioactivity remaining in the quenched reaction medium after phosphate extraction as phosphomolybdate with 2-butanol/benzene (1:1, v/v), as described by de Meis and Carvalho [28].

Gel electrophoresis

SDS/PAGE was performed according to the method of Weber and Osborn [29] with 7.5 % (w/v) acrylamide. The samples for electrophoresis were prepared as follows: 1 mg/ml of SR vesicles modified with PGO in the absence and in the presence of 0.5 mM ATP were incubated in 20 mM Mops, pH 6.8, 80 mM KCl containing 0.01 mg/ml of trypsin for 10 min at room temperature, and the reaction was stopped with 0.02 mg/ml of trypsin inhibitor. Fluorography was performed after SDS/PAGE.

Peptide sequencing

For peptide sequencing SR vesicles were modified with 2 mM PGO in the presence and absence of 0.5 mM ATP. After 1 h, the reaction was quenched with 4 % trifluoroacetic acid (TFA; final concentration at 4 °C). After quenching, samples were centrifuged at 12000 g for 15 min at 4 °C and washed with 1 % TFA. Hydrolysis of the pellet was done in 0.06 M HCl in glass ampoules under vacuum for 16 h at 110 °C. The hydrolysate was lyophilized and solubilized in 0.1 % TFA. The peptide mixture was subjected to HPLC with an ODS-2D reverse-phase column (0.46 cm $\times$ 25 cm) equilibrated with solvent A (0.1 % TFA in water), and detection at 225 nm. The column was developed with solvent B (0.1 % TFA in acetonitrile) at a flow rate of 1 ml/min with the following gradient: 10 to 55 % solvent B in 40 min, 55 % B for 5 min and 55 to 10 % B in 20 min. Fractions (0.5 ml) were collected and assayed for radioactivity content.

The fractions were labelled with 30 μ M fluorescamine in a buffer containing 0.2 M borate, pH 9.0, and electrophoresed in 16.5 % polyacrylamide according to Schägger and Von Jagow [30]. The gel was photographed under UV light to detect the fluorescamine-labelled peptides separated in the gel. These peptides were then electrotransferred on polyvinylidene difluoride (PVDF) membranes basically as described by Matsudaira [31]. The applied voltage was 80 V for 45 min. The PVDF membrane was then carefully rinsed in water and excited under UV light to detect the fluorescent peptides, these areas were excised and further processed for amino acid sequencing in an Applied Biosystems automated sequencer model 177A.

Calcium uptake and reversal of the pump

Ca²⁺ uptake experiments were performed at 25 °C with a reaction mixture containing 0.25 mg/ml native SR or PGO-modified SR, 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1.1 mM CaCl₂, 1 mM EGTA and 5 mM acetyl phosphate. Quenching of the Ca²⁺ uptake reaction at serial times was obtained by addition of 10 mM LaCl₃, 20 mM Mops, pH 6.8, and 80 mM KCl. An aliquot (0.065 mg of vesicles) was filtered and washed with 5 ml of the quenching solution.

SR vesicles were actively loaded with calcium using acetyl phosphate as substrate. The reversal of the catalytic cycle was promoted by addition of 10 mM EGTA, 1 mM P_i and 2 mM ADP. Aliquots were filtered at different time periods. A control experiment was performed by addition of 10 mM EGTA to SR vesicles loaded with calcium.

RESULTS AND DISCUSSION

PGO binding

PGO is a well known reagent of arginine residues [32]. The guanidino group of an arginyl residue reacts with one molecule of PGO to form an intermediate which irreversibly reacts with a

Figure 1 Effect of PGO binding on ATPase activity

Calcium ATPase was modified with 2 mM PGO in a medium containing 2 mg/ml SR, 30 mM Mops, pH 8.0, 80 mM KCl and 1 mM EGTA, in the presence and in the absence of 0.5 mM ATP. An aliquot was withdrawn at different times to measure PGO binding (triangles) and ATPase activity (circles) after PGO reaction in the presence (closed symbols) and absence (open symbols) of 0.5 mM ATP. PGO binding to specific sites (■) was calculated as the difference between PGO bound in the presence (▲) and in the absence (△) of ATP in the reaction medium. The error bar is calculated from five experiments.

Figure 2 Autoradiography of PGO labelling

Autoradiography of SDS/PAGE of Ca^{2+} -ATPase labelled with 2 mM [^{14}C]PGO in the absence (lanes 1 and 2) and in the presence of 0.5 mM ATP (lane 3). Lanes 2 and 3 show a controlled tryptic digestion at T_1 of PGO-modified ATPase in the absence and in the presence of ATP, respectively. The molecular-mass markers (kDa) are indicated on the right-hand side. The amount of protein in each lane was 0.015 mg.

second molecule of PGO to form an acid-stable adduct with the stoichiometry of 2:1 [33].

Incubation of SR vesicles with PGO at pH 8.0 results in binding of PGO, accompanied by loss of ATPase activity (Figure 1). When about 50 nmol of PGO were bound per mg of protein an almost complete ATPase inhibition was attained at a reaction time of 60 min. However, if the reaction medium contained 0.5 mM ATP the enzyme was protected against reaction with PGO, and about 63 % of ATPase activity was retained. Therefore if PGO binds to sites which are not protectable by ATP it will cause an inhibition of about 27 % of the activity. About 55 % of the remaining activity (63 %) will be inhibited by the binding of PGO to ATP-protectable sites, and some residual activity (about 8 %) will be totally independent of PGO binding (Figure 1). Therefore there is an important inhibitory effect caused by the binding of PGO to ATP-protectable sites, and this corresponds to about 18 nmol of PGO/mg of protein (Figure 1). Taking into account the molecular mass of the Ca^{2+} -ATPase and that its

Figure 3 Effect of PGO on ATP binding

(A) Effect of the reaction time with 2 mM PGO on the extent of ATP binding to PGO-modified ATPase in the presence (●) and absence (○) of 0.5 mM ATP. (B) Extent of [γ - ^{32}P]ATP binding to native ATPase (●) and PGO-modified ATPase (○). The ATP concentration used to assay ATP binding was 20 μM . The level of PGO binding to specific sites in these samples was 12 nmol/mg of protein. Results of two experiments are included in the Figures.

content in this membrane fraction is about 85 % we can calculate a stoichiometry of 2.3 molecules of PGO bound to one molecule of ATPase, at ATP-protectable sites. This figure would account for only one arginine residue modified by PGO. However, based on the total amount of active ATPase (4 nmol of phosphoenzyme/mg of protein) we can calculate a stoichiometry of 4.5 mol of PGO/mol of active ATPase. If each arginine residue reacts to completion, the first value would account for one arginine residue, and the second one would account for two arginines. On the other hand, if under our experimental conditions one or more arginines do not react to completion the number of reactive residues would be even bigger. Taking into account all these possibilities one can estimate a range of one to four possible arginine residues which can react with PGO to inhibit ATP binding.

[^{14}C]PGO-labelled Ca^{2+} -ATPase was digested with trypsin to cause a single cleavage at Arg⁵⁰⁵ producing A and B fragments [34] which can be well separated by SDS/PAGE. Figure 2 shows the SDS/PAGE of trypsin-digested Ca^{2+} -ATPase labelled with [^{14}C]PGO and its corresponding autoradiography. It can be seen that PGO is distributed in both A and B fragments (Figure 2, well 2). In a parallel experiment ATP was included in the medium of PGO labelling to protect the enzyme against PGO binding to some of the sites. In this case PGO label is mainly found in the A fragment (Figure 2, well 3). This result indicates that the arginine residues which are protected by ATP binding reside in the B fragment.

Nucleotide binding

We have seen in Figure 1 that PGO binding is partially prevented by the presence of ATP. In the same way modification with PGO results in a drastic decrease in the extent of ATP binding, as is shown in Figure 3. However, we can not achieve full inhibition of ATP binding since 1 nmol of ATP/mg remained bound at high PGO concentrations. Thus, from Figure 3(B) we can see that at saturating ATP concentrations a 75 % inhibition of ATP binding is reached when 12 nmol of PGO are bound per mg of protein.

In order to study the specificity of PGO modifications with respect to the nucleotide-binding inhibition, we have used ATP analogues such as TNP-AMP and TNP-ATP both of which bind with high affinity to the nucleotide-binding site of the Ca^{2+} -ATPase [26,35–40]. We have found that the inhibition of TNP-AMP binding to PGO-modified Ca^{2+} -ATPase is more pro-

nounced than that of TNP-ATP, i.e. 40 % and 17 % inhibition respectively (results not shown), but in both cases it is lower than the inhibition for ATP binding (about 75 % inhibition).

FITC is a well-characterized inhibitor of ATP binding by covalent modification of the B fragment at Lys⁵¹⁵ [6]. It lacks an anionic moiety compared with the nucleotide molecule which has the phosphate groups. Therefore its high affinity is probably due to a hydrophobic interaction through its aromatic rings to the purine base recognition pocket in the nucleotide-binding site. We obtained no significant inhibition of FITC labelling by previous PGO modification. The extent of FITC incorporation was 5.8 and 5.5 nmol of FITC/mg of SR for native Ca²⁺-ATPase and PGO-modified Ca²⁺-ATPase, respectively. In order to check that FITC is not labelling other different sites we performed SDS/PAGE of tryptic fragments from native and PGO-modified Ca²⁺-ATPase. It was found that FITC labels only B tryptic fragment in both samples, indicating that FITC labelling is not affected by the presence of PGO in its proximity. To exclude the possibility that FITC binding could release bound PGO from the enzyme we performed the same experiment but using [¹⁴C]PGO. We found PGO in both A and B fragments, therefore FITC binding does not displace PGO from its sites (results not shown).

The opposite experiment consists of labelling the Ca²⁺-ATPase with FITC before reaction with [¹⁴C]PGO. In this case prelabelling with FITC inhibited the PGO incorporation by 23.8 % ± 3.5 (mean values of five experiments) which is slightly lower than the value with ATP (36.5 % ± 5.5), so that the level of PGO bound to FITC-SR was 38.1 ± 2.7 nmol/mg and to SR in the presence of ATP was 31.8 ± 3.5 nmol/mg at 60 min (see also Figure 1). [¹⁴C]PGO labelling of B fragment was highly reduced, while labelling of A fragment was not affected in the Ca²⁺-ATPase prelabelled with FITC, as seen by autoradiography of SDS/PAGE of these samples digested with trypsin (results not shown). In this case FITC resides in the B fragment while PGO resides mainly in the A fragment. This observation clearly shows that FITC prevents the binding of PGO to B fragment in a similar way to when the PGO reaction is performed in the presence of ATP (Figure 3).

We have shown that the PGO reaction does not interfere with the reaction of FITC with Lys⁵¹⁵; however, FITC labelling prevents PGO reaction. This observation shows that the sequence of labelling is crucial in determining the accessibility of PGO to its reactive site. It is feasible that FITC obstructs the access of PGO to a region in which PGO binds more deeply than FITC. In this case FITC would block the entrance of PGO to the nucleotide-binding pocket. Conversely, PGO would not affect FITC binding because FITC would bind outside the PGO binding cleft. Another possibility to take into consideration is that FITC binding to Lys⁵¹⁵ could alter the ionic charge of the surroundings, increasing the positive charge over arginine residues and consequently inhibiting the reaction with PGO.

Calcium binding, phosphorylation with ATP and P_i and ATP synthesis

In order to know whether other partial steps, apart from ATP binding, participate in the ATPase inhibition we studied the other partial steps of the catalytic cycle such as calcium binding, phosphorylation with ATP and inorganic phosphate, and ATP synthesis in the reversal of the catalytic cycle. We have found that neither the affinity to calcium nor the total amount of calcium bound are affected by PGO. Similarly, the equilibrium of phosphoenzyme formation by inorganic phosphate is the same in native Ca²⁺-ATPase and in PGO-modified Ca²⁺-ATPase (results not shown). However, enzyme phosphorylation with ATP is

Figure 4 Effect of PGO binding on phosphorylation by ATP

Time course of PGO reaction is plotted versus the level of phosphorylation with ATP. Phosphorylation by [γ -³²P]ATP was assayed on ATPase that had been modified with PGO in the presence (●) and absence (○) of 0.5 mM ATP. Phosphorylation was initiated by adding 1 mM [γ -³²P]ATP to 1 mg/ml SR in 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂. The data correspond to mean values of two experiments with an error within 12%.

Table 1 Effect of PGO binding on ATP synthesis

Phosphoenzyme was formed with [³²P]P_i in leaky vesicles before and after PGO modification. ATP synthesis was promoted by addition of 5 mM CaCl₂ and 2 mM ADP to the phosphoenzyme. Mean values are within 7% error from two experiments.

Time (min)	Phosphoenzyme (nmol/mg)		ATP (nmol/mg)	
	– PGO	+ PGO	– PGO	+ PGO
0	2.80	2.50	0.0	0.0
1	0.96	1.50	0.53	0.01
10	0.16	0.74	0.80	0.10

inhibited in PGO-modified Ca²⁺-ATPase, whereas the presence of ATP in the PGO modification medium protects the ability of the enzyme to be phosphorylated by ATP (Figure 4).

Reversal of the catalytic cycle can be accomplished by adding high ADP concentrations to appropriate phosphorylated intermediates, the binding of ADP to the enzyme being a necessary requisite. To investigate the accessibility of ADP to the enzyme we triggered calcium release from actively preloaded vesicles by addition of 2 mM ADP, 1 mM EGTA and 1 mM P_i. In native vesicles calcium is released, accounting for ADP interaction with the enzyme, and consequently the reversal of the catalytic cycle leads to ATP synthesis. However, in PGO-modified vesicles there is no calcium release promoted by ADP addition to calcium-preloaded vesicles (results not shown).

Transfer of phosphate from phosphoenzyme to ADP can result in ATP synthesis under certain experimental conditions [41]. Phosphorylation with inorganic phosphate yields essentially the same level of phosphoenzyme in the presence and in the absence of PGO (Table 1). Addition of 2 mM ADP and 20 mM calcium results in ATP synthesis and phosphoenzyme decay (Table 1). However, Ca²⁺-ATPase modified with PGO is not able to transfer the phosphate from the enzyme to ADP, as

Figure 5 Kinetics of ATPase inhibition by PGO

(A) Effect of PGO concentration on the ATPase activity. The concentrations of PGO assayed were 0.5 mM (○), 1 mM (□), 1.5 mM (△), 2 mM (▽), 2.5 mM (◇), 3 mM (▲), and 6 mM (●). Each curve was repeated at least twice, and representative data are shown. (B) Inhibition rate constant calculated for first-order kinetics corresponding to the experimental data shown in (A). The continuous line corresponds to the fitting to eqn. (4) with the following constants: $K_I = 2.2 \text{ mM}^{-1} \cdot \text{min}^{-1}$ and $k = 0.023 \text{ min}^{-1}$.

can be seen in Table 1 where no ATP synthesis derived from phosphoenzyme decay is detected.

From all these results it could be proposed that the binding of PGO to these sites (which are not protectable by ATP) could modify, to a limited extent, the transfer of phosphate from ATP to the ATPase to form the phosphoenzyme.

Kinetic mechanism of enzyme inhibition

The kinetics of the inhibition of ATP hydrolysis was studied with the aim of elucidating the mechanism of PGO inhibition.

Ca^{2+} -ATPase was incubated with different concentrations of PGO. An aliquot was withdrawn at different time intervals to assay the ATPase activity in a medium where the PGO concentration was diluted at least 100-fold to assure that the inhibition reaction was stopped. Under these conditions the ATPase activity is highly dependent on the PGO concentration (Figure 5A). Below 0.5 mM PGO the ATPase activity was almost totally preserved during up to 30 min of preincubation time with PGO. However, when the PGO concentration was raised to 6 mM about 90% inhibition was achieved after a reaction time of 30 min (Figure 5A).

The ATPase inhibition follows pseudo-first-order kinetics within the PGO concentration range assayed. An apparent rate

constant can be calculated from the slope of the curves of log (activity) versus time at each PGO concentration used in the preincubation medium. The calculated rate constants are plotted versus PGO concentration in Figure 5(B).

The dependence of ATPase activity with the concentration of PGO at an incubation time of 30 min was studied at increasing ATP concentrations in the reaction medium. The presence of ATP protected against the inactivation process (results not shown), and this may indicate the possibility of competition between ATP binding and PGO modification.

The kinetic mechanism of the inhibition of the ATPase activity should include these features: PGO binding to free enzyme, and a total amount of two molecules of PGO bound per molecule of enzyme, as it is shown in eqn. (1):

where K_I is the equilibrium dissociation constant of EI, and k is the rate constant for the binding of the second inhibitor molecule. In order to assay the activity of the enzyme an aliquot was withdrawn from the incubation medium when the inhibition process was taking place. Therefore the active enzyme, E_A , will be formed by E and EI ($[\text{E}_A] = [\text{E}] + [\text{EI}]$) and the velocity of the reaction of catalysis will depend on the concentration of E_A . Thus,

$$v = -d[\text{E}_A]/dt = k[\text{I}][\text{E}_A] \quad (2)$$

substituting $[\text{E}_A]$ in the expression of K_I , we obtain

$$-d[\text{E}_A]/dt = k[\text{I}]^2[\text{E}_A]/(K_I + [\text{I}]) = k_{app}[\text{E}_A] \quad (3)$$

$$k_{app} = k[\text{I}]^2/(K_I + [\text{I}]) \quad (4)$$

integrating eqn. (3) it follows that:

$$[\text{E}_A] = [\text{E}_T]e^{-k_{app} \cdot t} \quad (5)$$

Therefore the reaction of inactivation follows a first-order kinetic (eqn. 5), its rate constant, k_{app} , being dependent on $[\text{I}]$ (eqn. 4). The experimental data of k_{app} versus PGO concentration has been fitted to eqn. (4) and plotted as a continuous line in Figure 5(B), with $K_I = 2.2 \text{ mM}$ and $k = 0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$.

In this mechanism the intermediates with bound PGO would be phosphorylated by inorganic phosphate, but would not bind nucleotides, thus impeding ATP synthesis, and consequently the reversal of the catalytic cycle.

Taking into account the mechanism of the PGO reaction with arginine (eqn. 1) we can assume that binding of two PGO molecules might represent binding to the same site in the enzyme, and definitively the participation of only one arginine residue is enough to match the experimental results with the proposed mechanism. Similar behaviour was observed in the calcium pump from cardiac SR, and from skeletal muscle using PGO and butanedione respectively as reagents for arginyl residues [20,42]. In both cases the inhibitor was shown to inactivate the enzyme in a pseudo-first-order reaction by binding to a single arginine at the nucleotide-binding site.

Localization of PGO reactive sites in the Ca^{2+} -ATPase

To go further in the characterization of the reactive arginine residue a partial hydrolysis was carried out on the Ca^{2+} -ATPase prelabelled with [¹⁴C]PGO. A mild acid hydrolysis can be achieved by incubating with 0.06 M HCl for 16 h. Under these conditions preferentially peptidic bonds at aspartic acid residues become hydrolysed [43]. After purification of the radioactive peptides only two majors bands were visible by SDS/PAGE with molecular masses of 10600 Da (band I) and 4000 Da (band II).

These bands were extracted and processed for automated amino acid sequencing. We were able to sequence the first 5–7 amino acid residues. Band I yields the sequence DRKSMS and band II the sequence RKSQRI. These sequences can be unambiguously assigned to the amino acid sequence of the Ca^{2+} -ATPase. In this way band II sequence matches with a segment beginning at Arg¹³⁴ and would contain around 40 amino acid residues according to the molecular mass determined from SDS/PAGE. This peptide has the following sequence: RKSQRIKARDIV-PGDIVE VAVGDKVPADIRILSIKSTTL. This segment contains a total of four arginine residues and it is part of tryptic subfragment A2. The previous amino acid residue happens to be an aspartic acid, hence an Asp-Arg bond has been hydrolysed. On the other hand band I sequence begins at Asp⁴⁹⁰ and would contain about 106 residues, with the following sequence: DRKSMSVYCSPAKSSRAAVGNKMFVKGAPEGVIDRC NYVRVGTTTRVPMTPGVKEKILSVIKEWGTGRDTRLRLALATRDTPPKREEMVLDDSSRFMEYETDLTFVG. Fifteen residues belong to tryptic subfragment A1 and the remaining residues belong to tryptic fragment B, where eight arginines belong to the latter. This fragment would contain the arginine residue involved in ATP binding since this residue resides only in the B fragment (Figure 2).

Since the labelling which is not protectable by ATP was identified in tryptic fragment A (peptide from Met¹ to Ser⁵⁰⁴) then the peptide of Arg¹³⁴ contains the arginine residues not protectable by ATP. Although peptide starting at Asp⁴⁹⁰ is located also in the A fragment, it spans to the B fragment (peptide from Arg⁵⁰⁵ to Lys¹⁰⁰¹). Considering the molecular mass estimated by SDS/PAGE of the sequenced peptides the first peptide could be Arg¹³⁴–Leu¹⁷⁴ and it would belong to A2 tryptic subfragment. The second peptide could be Asp⁴⁹⁰–Gly⁵⁹⁵ and it would contain a small portion of A1 tryptic subfragment and the main part would belong to the B fragment (Figure 6). Since ATP-protectable labelling is located only in the B fragment we can discard the A1 sequence (Asp⁴⁹⁰–Arg⁵⁰⁵). Consequently, sequence Ala⁵⁰⁶–Gly⁵⁹⁵ can be assigned to the peptide which contains some arginine residues which are influenced by the binding of ATP at its site.

Although the stoichiometry of PGO binding and the kinetic mechanism of the inhibition agrees with the participation of one arginine residue (two molecules of PGO) we can not exclude the possibility of the involvement of more than one residue in the ATPase inhibition. In this fragment (Ala⁵⁰⁶–Gly⁵⁹⁵) there are a total of eight arginine residues; all of them are possible candidates to be modified by PGO. Unfortunately it has not been possible to determine the modified residue since Edman degradation releases PGO.

An important feature of the Ca^{2+} -ATPase is its ability to bind nucleotides at the cytoplasmic side of the protein. A number of amino acid residues from different parts of the molecule might participate in configuring the nucleotide-binding pocket. The fragment corresponding to band I (Figure 6) contains some residues which are known to be involved in ATP binding: FITC binds to Lys⁵¹⁵, and TNP- N_3 -AMP and other ATP derivatives covalently label Lys⁴⁹² [12,44]. This residue, Lys⁴⁹², is also labelled with pyridoxal 5'-phosphate [45] and with adenosine triphosphopyridoxal in the absence of calcium [46]. Glutaraldehyde cross-links Lys⁴⁹² with Arg⁶⁷⁸ in the nucleotide site, indicating the proximity of these residues [11].

Site-directed mutagenesis has produced valuable information on the amino acids actually involved in the nucleotide site. Some amino acids included in band I have been mutated to inhibit phosphoenzyme formation from ATP, such as fragment Lys⁵¹⁵–Glu⁵¹⁹ and fragment Asp⁶⁰¹–Arg⁶⁰⁴. All these modi-

Figure 6 Diagram of the localization of the sequenced peptides on the primary structure of the Ca^{2+} -ATPase

T_1 and T_2 are the first and second cleavage sites of trypsin which yield A and B fragments, and A_1 and A_2 subfragments, respectively. The sequenced peptides are marked and scaled on the total length of the ATPase (upper drawing). A tentative scheme of ATPase folding is shown in the lower part of the drawing, based on McIntosh [11], where band I is shown in a thicker black line containing the nucleotide-binding site.

fications inhibit ATP binding, suggesting that these amino acid residues are somehow involved in the nucleotide pocket.

On the other hand monoclonal antibodies raised against different sequences of the Ca^{2+} -ATPase have revealed that binding to fragments Asn⁵¹⁰–Lys⁵¹⁵ or Asp⁶⁵⁹–Glu⁶⁶⁸ inhibits ATPase activity but no effect is observed when there is binding to fragment Leu⁵⁸⁰–Glu⁵⁸⁸ or Val³³³–Ser³³⁸. An important fact is that fragment Asn⁵¹⁰–Lys⁵¹⁵ is probably close to fragment Asp⁶⁵⁹–Glu⁶⁶⁸ but fragment Leu⁵⁸⁰–Glu⁵⁸⁸ is located more distant to fragments Asn⁵¹⁰–Lys⁵¹⁵ and Asp⁶⁵⁹–Glu⁶⁶⁸ [47].

Considering the positive charge of an arginine residue we can argue that this residue might be involved in nucleotide binding by an ionic bond with the phosphate groups. Since binding of ADP and even TNP-AMP are also inhibited, the most probable interaction would be with the α -phosphate of the nucleotide. Nevertheless steric obstruction can not be ruled out because of the molecular size of the adduct formed between an arginine residue and two molecules of PGO.

From all these experiments, and although it should be remembered that we have not obtained an absolute proof, it can be concluded that the protein fragment affected by binding of PGO is located right at the ATP-binding cleft. It seems reasonable that fragment Asp⁴⁹⁰–Glu⁶⁸⁰ makes a hairpin loop in which ATP interacts preferentially with fragments Asp⁴⁹⁰–Lys⁵¹⁵ and Asp⁶⁵⁹–Glu⁶⁸⁰. Peptide Asp⁴⁹⁰–Gly⁵⁹⁵ (band I) would correspond to half of the loop, containing an arginine residue in a deeper position than Lys⁵¹⁵ in the tertiary structure of the protein, and this residue participates in nucleotide binding.

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