The Ca^{2+} -transporting ATPases of rabbit and trout exhibit different pH- and temperature-dependences

Eduardo N. CHINI, Frederico G. S. DE TOLEDO, Mauro C. ALBUQUERQUE and Leopoldo DE MEIS*

Departamento de Bioquímica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brazil

The phosphorylation of the trout sarcoplasmic-reticulum Ca^{2+} -ATPase by P_i differs in its temperature- and pH-dependence from the rabbit ATPase. In the trout enzyme, the apparent affinity for P_i and maximum phosphoenzyme values do not vary over a pH and temperature ranges that have a pronounced effect on the rabbit enzyme. The lack of temperature-dependence for phosphorylation is observed at pH 6.8. At pH 8.0, the temperature profile for phosphorylation of the trout enzyme resembles that of the rabbit at pH 6.8. The rabbit ATPase is no longer phosphorylated by P_i after solubilization with the detergent C_{12}E_9 . In contrast, the trout enzyme can be phosphoryl-

ated by P_i after solubilization with C_{12}E_9 , and the same levels of phosphoenzyme were obtained with the soluble and membrane-bound ATPase at both 0° and 25 °C. In the range of 0–20 °C, the rates of ATP synthesis and of Ca^{2+} uptake by the trout ATPase are less temperature-dependent than for the rabbit enzyme. However, both isoenzymes catalyse ATP hydrolysis with similar temperature-dependences. The results raise the possibility that protonation of specific amino acid residues may contribute to the lack of temperature-dependence for phosphorylation of the trout Ca^{2+} -ATPase.

INTRODUCTION

The Ca^{2+} -ATPase (EC 3.6.1.38) of sarcoplasmic-reticulum membranes catalyses both the hydrolysis and synthesis of ATP [1]. The synthesis of ATP is initiated by phosphorylation of the ATPase by P_i [1–4] and can occur both in the presence and in the absence of a transmembrane Ca^{2+} gradient [5–7]. For the rabbit skeletal-muscle Ca^{2+} -ATPase this reaction varies greatly, depending on the pH and temperature of the assay medium [1,3–5,8–11]: the K_m for P_i increases from 1.5 mM to over 10 mM when the pH of the medium is raised from 6.0 to 7.5, and the phosphoenzyme formation is impaired when the temperature decreases from 20 °C to 0 °C. The effects of temperature and pH are abolished when organic solvents [9,10], methylamines [12] or saccharides [13] are added to the assay media. Lipids and detergents also alter the properties of the Ca^{2+} -ATPase [1,14,15]. After solubilization with detergents, the rabbit enzyme becomes unstable if incubated in the absence of Ca^{2+} and is denatured in a few seconds [14].

In this work we compare the effects of pH, temperature and detergent C_{12}E_9 on the Ca^{2+} -ATPase isolated from rainbow trout with those on that isolated from rabbit skeletal muscle. It is shown that the phosphorylation of the trout ATPase by P_i varies little when the pH is raised from 6.3 to 7.5, when the temperature is decreased from 20 °C to 0 °C, or when the enzyme is solubilized by the detergent C_{12}E_9 .

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles derived from rabbit skeletal muscle and from rainbow trout (*Salmo gairdneri*) dorsolateral muscles were prepared as described by Eletr and Inesi [16]. The fish used were adapted to the temperature range of 10–18 °C. Protein concentrations were determined as described by Lowry et al. [17], with BSA as standard. In both preparations the Ca^{2+} -dependent ATPase activity was not inhibited by 1 mM ouabain,

but was totally abolished by 1 mM ammonium vanadate, a specific inhibitor of $\text{E}_1\text{--E}_2$ enzymes.

SDS/PAGE was performed on a 6–15 % polyacrylamide gradient gel and stained with Coomassie Blue [18]. Both preparations revealed a major band of 110 kDa corresponding to the Ca^{2+} -ATPase (Figure 1).

Phosphorylation of the Ca^{2+} -ATPase by [^{32}P] P_i was determined by using Millipore filters [19]. Filters were washed with 15 × 5 ml of 0.1 M HCl/2 mM P_i . The phosphorylation reaction was quenched by addition of ice-cold trichloroacetic acid (10 %, w/v) containing 2 mM non-radioactive P_i . Blank tubes were prepared by quenching the enzyme with the trichloroacetic acid solution before addition of radioactive P_i .

Ca^{2+} uptake was assayed with $^{45}\text{CaCl}_2$ and Millipore filters [19]. Potassium oxalate was used as a Ca^{2+} -precipitating agent.

ATP hydrolysis was determined by the colorimetric method of Fiske and Subbarow [20].

Synthesis of ATP from ADP and [^{32}P] P_i was measured as previously described [19], by using molybdate and butan-2-ol/benzene to extract the excess [^{32}P] P_i from the medium.

Both ATPases were solubilized by diluting the protein to 1 mg/ml in an ice-cold medium containing 50 mM Mops/Tris, 0.1 mM CaCl_2 and 4 mg of C_{12}E_9 detergent/ml. The enzyme was solubilized immediately before the experiment, and phosphoenzyme measurements of the soluble enzyme were obtained by the centrifugation method [19].

[^{32}P] P_i was obtained from the Brazilian Institute of Atomic Energy and purified as previously described [19].

All values shown are averages of 2–4 experiments.

RESULTS

Phosphorylation by P_i

As described for the rabbit Ca^{2+} -ATPase [1,6], the phosphorylation of the trout ATPase by P_i is activated by Mg^{2+} and inhibited by Ca^{2+} (results not shown). Different temperature-dependences are observed, depending on the animal species used

* To whom correspondence should be addressed.

Figure 1 Protein composition of (a) marker, (b) rabbit vesicles and (c) trout vesicles

The electrophoresis was performed in 6–15% gradient SDS/polyacrylamide gel.

(Figure 2). At pH 6.8 the rabbit ATPase phosphoenzyme level increases as the temperature is raised from 0 °C to 20 °C, after which it remains constant up to 45 °C. The trout Ca^{2+} -ATPase exhibits a higher level of phosphoenzyme at 0 °C, and there is a very little increase when the temperature is raised to 20 °C. Above 30 °C the level of phosphoenzyme decreases, due to thermal inactivation. The ATPase activity of the trout enzyme is impaired when the vesicles are preincubated for 30 min at 35 °C in a medium with the same composition as that used for measuring phosphorylation by P_i . This is not observed with the rabbit enzyme (Figure 3). The preincubation time needed for half-maximal inactivation of the trout ATPase decreases from 20 min to 30 s as the temperature is raised from 35° to 40 °C. A semi-logarithmic plot of phosphoenzyme level versus the reciprocal of the absolute temperature from 0° to 30 °C (Figure 2b) exhibits only a single slope for the trout Ca^{2+} -ATPase, whereas rabbit enzyme reveals a break between 18 °C and 22 °C [1,8,9]. For the rabbit ATPase it has been shown previously that the temperature-dependence is related to a change in the maximum phosphoenzyme levels and not to a change in the K_m for P_i [8].

Figure 2 Temperature dependence for phosphorylation by P_i

(a) The reaction medium contained 50 mM Mops/Tris buffer (pH 6.8), 10 mM MgCl_2 , 2 mM EGTA, 3 mM $^{32}\text{P}\text{P}_i$ and 0.3 mg/ml trout (▲) or rabbit (●) vesicles. The reactions at different temperatures were started by addition of the protein and stopped after 30 s. (b) Semi-logarithmic plot of equilibrium phosphoenzyme (E-P) level versus the reciprocal of the absolute temperature.

Figure 3 Thermal inactivation of ATPase activity

Rabbit (○) or trout (●) vesicles at a concentration of 3 mg/ml were incubated at 35 °C in a medium containing 50 mM Mops/Tris (pH 7.0) and 2 mM EGTA. At the times indicated, samples were diluted in a medium at 30 °C for ATPase-activity assay containing 50 mM Mops/Tris buffer (pH 7.0), 0.13 mM CaCl_2 , 10 mM MgCl_2 , 1 mM ATP, 5 mM potassium oxalate and 0.1 mM EGTA. The final protein concentration was 0.03 mg/ml. In the Figure, 100% activity corresponds to 1.5 and 3.0 μmol of P_i /min per mg for the rabbit and trout ATPase respectively.

This was confirmed by using the conditions described in Figures 2 and 4. At 12°, 17° and 35 °C the K_m values for P_i were 2.7, 2.5 and 2.8 mM respectively, and the maximum levels of phosphoenzyme were 0.85, 1.0 and 1.9 μmol /g of protein. At 17 °C, the K_m value for the trout ATPase is about half that measured with the rabbit ATPase (Table 1). Neither the K_m for P_i nor the maximum level of phosphoenzyme for the trout ATPase varies in the temperature range of 12–20 °C (Table 1).

The apparent K_m for P_i of the rabbit ATPase varies greatly depending on the pH of the medium, increasing from 1.5 mM at pH 6.0 to over 10 mM at pH 7.5 [1,6,11]. At 20 °C, this pronounced pH-dependence is not observed in the trout ATPase (Figure 4 and Table 1). However, the temperature-dependence of the phosphoenzyme formation from P_i by the trout ATPase is altered by the pH of the assay medium. At pH 8.0, the semi-logarithmic plot of phosphoenzyme level versus the reciprocal of the absolute temperature shows a break in the range of 18–22 °C which is not observed at pH 6.8 (Figure 5). At pH 8.0 the two slopes are similar to those observed for the rabbit enzyme at

Figure 4 K_m for P_i at different pH values

The reaction was started at 20 °C by addition of trout vesicles to a medium containing 10 mM MgCl_2 , 2 mM EGTA, $[\text{P}_i]$ at different concentrations and 50 mM Mops/Tris buffer at pH 6.3 (●), pH 7.0 (▲) or pH 7.5 (◆). The final protein concentration was 0.3 mg/ml. The reaction was quenched after 30 s.

Table 1 Temperature- and pH-dependence for phosphorylation by P_i (trout ATPase)

The reaction was started by addition of trout vesicles to a final concentration of 0.3 mg/ml and stopped after 30 s. The reaction medium consisted of 2 mM EGTA, 50 mM Mops/Tris buffer plus 10 mM MgCl_2 and diverse $[\text{P}_i]$ concentrations (for measurements of K_m for P_i) or 3 mM $[\text{P}_i]$ and diverse Mg^{2+} concentrations (for measurements of K_m for Mg^{2+}). —, not measured. E-P, phosphoenzyme.

Temp. (°C)	pH	P_i		Mg^{2+}	
		K_m (mM)	E-P (μmol/g of protein)	K_m (mM)	E-P (μmol/g of protein)
0	7.0	—	—	2.31	1.25
12	7.0	1.26	2.78	1.36	1.81
17	7.0	1.14	2.77	—	—
20	7.0	1.52	2.67	1.20	1.56
20	6.3	0.63	2.36	1.37	2.32
20	7.0	1.52	2.67	1.20	1.56
20	7.5	1.82	2.02	0.99	1.14

pH 6.8 (Figure 2b). Finally, the Mg^{2+} -dependence for phosphorylation of the trout ATPase shows a small change when the temperature or pH of the medium is modified (Table 1).

ATP synthesis

Synthesis of $[\text{P}_i]\text{ATP}$ is observed when vesicles previously loaded with Ca^{2+} are diluted in a medium containing Mg^{2+} , EGTA, $[\text{P}_i]\text{ATP}$ and ADP [1,2,21]. At temperatures below 20 °C, the rate of ATP synthesis measured with trout vesicles is faster than that with the rabbit vesicles (Figure 6).

Ca^{2+} -dependent ATP hydrolysis and Ca^{2+} uptake

The same temperature-dependence is observed for the rabbit and trout ATPase activities (Figure 7a). For both enzymes there is a 85% decrease in the Ca^{2+} -dependent activity when the temperature is lowered from 30 °C to 0 °C, without any break in the

Figure 5 Temperature-dependence of phosphoenzyme formation at different pH values

The reaction medium contained 50 mM Mops/Tris buffer at either pH 6.8 (●) or pH 8.0 (▲), 10 mM MgCl_2 , 2 mM EGTA, 3 mM $[\text{P}_i]\text{ATP}$ and 0.3 mg/ml trout vesicles. The reaction was started by addition of the protein and stopped after 30 s incubation at different temperatures.

Figure 6 Temperature-dependence of ATP synthesis

Trout (●) and rabbit (○) vesicles (0.05 mg/ml) were loaded with 0.2 mM CaCl_2 in a medium with 50 mM Mops/Tris buffer (pH 7.0), 10 mM MgCl_2 , 20 mM P_i and 5 mM ATP for 45 min at room temperature (trout) or 35 °C (rabbit) and then centrifuged at 40 000 g for 30 min. The pellet was resuspended in cold deionized water. The ATP-synthesis reaction was started by addition of vesicles to a medium containing 50 mM Mops/Tris buffer (pH 7.0), 10 mM MgCl_2 , 2 mM EGTA, 4 mM $[\text{P}_i]\text{ATP}$, 10 mM glucose, 0.02 mg/ml hexokinase and 0.2 mM ADP. The final concentration of vesicles was 0.057 mg/ml. Samples were quenched at 0.5, 1, 2 and 5 min of reaction, and the initial rate was calculated from the slope, after correction for the blank. In the Figure, 100% activity corresponds to 31.6 and 24.6 nmol of ATP synthesized/min per mg for the rabbit and trout ATPase respectively.

Arrhenius plot. A similar single profile was previously observed for the rabbit ATPase when the vesicles were treated with diethyl ether to prevent the increase in Ca^{2+} concentration in the vesicle lumen [22]. In order to maintain a low concentration of intravesicular free Ca^{2+} , a high concentration of oxalate was used in Figure 7(a). Under the same conditions, the rate of Ca^{2+} uptake (Figure 7b) reveals a different temperature profile in the two species, being faster for the trout vesicles at temperatures below 20 °C.

Solubilization of the Ca^{2+} -ATPase with C_{12}E_8

The phosphorylation reaction of the rabbit ATPase varies depending on the lipid environment in which the ATPase is

Figure 7 Temperature-dependence of (a) Ca^{2+} -dependent ATP hydrolysis and (b) Ca^{2+} uptake

(a) For the total ATPase activity the reaction-medium composition was 0.13 mM CaCl_2 , 1 mM ATP, 5 mM potassium oxalate, 10 mM MgCl_2 , 0.1 mM EGTA and 50 mM Mops/Tris buffer (pH 6.8). The Mg^{2+} -dependent ATPase activity was assayed in the same medium used for the total ATPase activity but without Ca^{2+} . The Ca^{2+} -dependent ATPase activity is the total ATPase activity minus the Mg^{2+} -dependent activity. The reaction was started by addition of trout (●) or rabbit (○) vesicles to a final concentration of 0.05 mg/ml and quenched after 1 or 2 min incubation at different temperatures. In the Figure, 100% activity corresponds to 1.4 and 3.0 μmol of P_i /min per mg for the rabbit and trout ATPase respectively. (b) The reaction medium was the same as described in (a) except for the presence of $^{45}\text{CaCl}_2$ instead of non-radioactive Ca^{2+} . The reaction was started by addition of 0.05 mg/ml of either trout (●) or rabbit (○) vesicles and stopped after 2 min incubation at different temperatures. In the Figure, 100% activity corresponds to 0.98 and 0.43 μmol of Ca^{2+} /min per mg for the rabbit and trout ATPase respectively.

Table 2 Phosphoenzyme in the presence or absence of C_{12}E_9

The reaction was started by addition of trout or rabbit ATPase, solubilized as described in the Materials and methods section, or with intact sarcoplasmic-reticulum vesicles (SRV), to a final concentration of 0.5 mg/ml and stopped after 30 s. The reaction was assayed in a medium containing 2 mM EGTA, 50 mM Mops/Tris buffer, 10 mM MgCl_2 and 3 mM $^{32}\text{P}_i$.

ATPase	Assay temp.	Control (SRV)		Solubilized with C_{12}E_9	
		0 °C	25 °C	0 °C	25 °C
Rabbit		0.12 ± 0.11	1.68 ± 0.01	0	0
Trout		1.87 ± 0.04	2.28 ± 0.27	1.98 ± 0.3	2.10 ± 0.1

embedded [1,14,15]. Table 2 shows that after solubilization with the detergent C_{12}E_9 the rabbit enzyme is not longer phosphorylated by P_i . In contrast, the levels of phosphoenzyme obtained with the trout before and after solubilization of the vesicles are the same both at 0° and at 25 °C (Table 2).

DISCUSSION

The role of the protein structure and the lipid environment in the temperature-dependence of the Ca^{2+} -ATPase is still an open question [1,22–25]. Vrbjar et al. [23] attribute the temperature adaptation of fish in cold water to a different lipid environment of sarcoplasmic-reticulum membranes. Dux et al. [24] have found that the tryptic cleavage of the carp sarcoplasmic-reticulum Ca^{2+} -ATPase leads to the formation of two fragments that differ from those of fragmented rabbit ATPase. They proposed that the dissimilarities between the carp and rabbit sarcoplasmic-reticulum vesicles may be related to different protein structures. Dean and Tanford [25] suggested that protein–lipid interaction is not responsible for the breaks seen in the Arrhenius plot for the Ca^{2+} -ATPase of rabbit and argue instead for temperature-dependence changes intrinsic to the polypeptide chain. This proposal is supported by the data of Table 2, showing that the detergent C_{12}E_9 has no effect on the temperature-dependence of phosphorylation of trout ATPase by P_i .

The temperature-dependence of the rabbit Ca^{2+} -ATPase (Fig-

ure 2b) is explained by changes in the equilibrium between the forms E_1 and E_2 of the enzyme [1,8,26,27]. At pH 6.8 this equilibrium probably does not vary with the temperature for the trout Ca^{2+} -ATPase (Figure 2b), since no temperature-dependence is observed for the phosphorylation reaction. However, when the pH is raised to 8.0, temperature-dependence becomes evident (Figure 5). This pH-determined temperature-dependence is not related to changes in the K_m for P_i or Mg^{2+} . Hence these data suggest that protonated states of some amino acid residues may influence the temperature-dependence of the equilibrium between the two forms E_1 and E_2 , and consequently the phosphorylation reaction.

At temperatures below 20 °C, net Ca^{2+} uptake by trout vesicles is faster than in rabbit vesicles (Figure 7), a phenomenon that could be of physiological relevance for the trout, because muscle relaxation is dependent on the Ca^{2+} -pumping ability of the sarcoplasmic reticulum.

The amount of Ca^{2+} accumulated by sarcoplasmic-reticulum vesicles is dependent on the rate of Ca^{2+} pumping and the rate of Ca^{2+} leakage. Recently it has been reported that the Ca^{2+} -ATPase may mediate a Ca^{2+} efflux that is blocked by phosphorylation of the enzyme by P_i [28–31]. The disparity between the temperature-dependence of Ca^{2+} uptake and that of ATPase activity (Figure 7) may perhaps be explained by the higher levels of phosphorylation found in the trout ATPase. This would decrease the leakage at low temperatures and therefore increase the amount of Ca^{2+} accumulated.

This work was partially supported by Financiadora de Estudos e Projetos (FINEP) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). E.N.C., F.G.S. de T. and M.C.A. are recipients of fellowships from CNPq. We are grateful to José and Luciana Aguirre for the kind donation of the fish and to Martha Sorenson for helpful discussion. E.N.C. expresses his gratitude to Dr. M. Fönseca.

REFERENCES

- de Meis, L. (1981) In *The Sarcoplasmic Reticulum: Transport and Energy Transduction: Transport in The Life Sciences*, vol. 2 (Bittar, E. E., ed.), John Wiley and Sons, New York
- Makinose, M. (1972) *FEBS Lett.* **25**, 113–115
- Masuda, H. and de Meis, L. (1973) *Biochemistry* **12**, 4581–4585
- Froud, R. and Lee, A. G. (1986) *Biochem. J.* **237**, 207–215
- de Meis, L. and Tume, R. K. (1977) *Biochemistry* **16**, 4455–4463
- de Meis, L. and Vianna, A. L. (1979) *Annu. Rev. Biochem.* **48**, 275–292

- 7 de Meis, L. (1989) *Biochim. Biophys. Acta* **973**, 333–349
- 8 Masuda, H. and de Meis, L. (1977) *J. Biol. Chem.* **252**, 8567–8571
- 9 de Meis, L., Martins, O. B. and Alves, E. W. (1980) *Biochemistry* **19**, 4252–4261
- 10 Champeil, Ph., Guillon, F., Venien, C. and Gingold, M. P. (1985) *Biochemistry* **24**, 69–81
- 11 Beil, F., Chak, D. and Hasselbach, W. (1977) *Eur. J. Biochem.* **81**, 151–164
- 12 de Meis, L. and Inesi, G. (1988) *J. Biol. Chem.* **263**, 157–161
- 13 Chini, E. N., Meyer-Fernandes, J. R. and Sola-Penna, M. (1991) *Z. Naturforsch.* **46C**, 644–646
- 14 Kanazawa, T. (1975) *J. Biol. Chem.* **250**, 113–119
- 15 Michelangeli, F., Grimes, E. A., East, J. M. and Lee, J. M. (1991) *Biochemistry* **30**, 342–351
- 16 Eletr, S. and Inesi, G. (1972) *Biochim. Biophys. Acta* **282**, 246–269
- 17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 18 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 19 de Meis, L. (1988) *Methods Enzymol.* **157**, 190–206
- 20 Fiske, C. H. and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- 21 Makinose, M. and Hasselbach, W. (1971) *FEBS Lett.* **12**, 271–272
- 22 Inesi, G., Millman, M. and Eletr, S. (1973) *J. Mol. Biol.* **81**, 483–504
- 23 Vrbjar, N., Simatos, G. A. and Keough, K. M. W. (1990) *Biochim. Biophys. Acta* **1030**, 94–100
- 24 Dux, L., Lelkes, G., Hieu, L. H. and Nemcsók, J. (1989) *Comp. Biochem. Physiol.* **92B**, 263–270
- 25 Dean, W. L. and Tanford, C. (1978) *Biochemistry* **17**, 1683–1690
- 26 Inesi, G., Lewis, D. and Murphy, A. J. (1984) *J. Biol. Chem.* **259**, 996–1003
- 27 Pick, U. and Karlisch, S. J. D. (1982) *J. Biol. Chem.* **257**, 6120–6126
- 28 Gerdes, V. and Moller, J. V. (1983) *Biochim. Biophys. Acta* **734**, 191–200
- 29 Gould, G. W., Colyer, J., East, J. M. and Lee, A. G. (1987) *J. Biol. Chem.* **262**, 7676–7679
- 30 de Meis, L., Suzano, V. A. and Inesi, G. (1990) *J. Biol. Chem.* **265**, 18848–18851
- 31 de Meis, L. (1991) *J. Biol. Chem.* **266**, 5736–5642