Intrinsic regulation of substrate fluxes and energy conservation in Ca^{2+}-ATPase

Leopoldo de Meis and Giuseppe Inesi*

Departamento de Bioquímica, Centro de Ciências da Saúde, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Rio de Janeiro 21910, Brazil, and **Department of Biochemistry, University of Maryland, Medical School, 660 West Redwood Street, Baltimore, MD 2101, USA*

Received 28 January 1985

The Ca^{2+}-ATPase of sarcoplasmic reticulum was utilized to demonstrate an intrinsic regulation of enzyme catalysis, whereby the ratio of forward and reverse flow is altered by the binding of Ca^{2+} and P_i to the enzyme. This is related to displacement of internal equilibria among intermediate enzyme · ligand complexes, independent of the overall equilibrium of the ATP $\rightleftharpoons$ ADP + P_i transformation. A very high energy conservation with a velocity of reverse flow approaching that of forward flow, was obtained by increasing the enzyme affinity for P_i in the presence of Me_2SO.

Energy conservation *ATPase* *Sarcoplasmic reticulum*

1. INTRODUCTION

The presence of enzymes in systems containing substrate and product concentrations far from equilibrium requires kinetic regulation of catalysis to insure that the substrate is utilized rapidly when product is required, and conserved when product is not required. Some enzymes, such as phosphorylase [1], are subjected to elaborate regulation by extrinsic enzyme cascades. Such an extrinsic regulation is also present in the Ca^{2+} transport ATPases of plasma membranes, which are subjected to kinetic regulation by calmodulin [2,3]. On the other hand, no extrinsic regulation has been found as yet for the ATPase of skeletal muscle SR. Operation of this enzyme as a Ca^{2+} pump involves formation of a phosphorylated intermediate by transfer of the ATP terminal phosphate to an aspartyl residue, followed by reduction in affinity and change in orientation of two calcium-binding domains in the enzyme. Following vectorial dissociation of Ca^{2+}, the phosphorylated intermediate undergoes hydrolytic cleavage and the enzyme recycles [4,5]. A Ca^{2+} concentration gradient across the SR membrane is thereby produced. Since the physiologic concentrations of ATP, ADP and P_i, and the Ca^{2+} concentration gradient across the SR membrane are not far from equilibrium [6,7], it could be inferred that net substrate utilization by the SR ATPase can be slowed down only when the appropriate equilibrium is approached. We provide here an experimental demonstration of intrinsic regulation of catalysis whereby in the absence of a transmembrane Ca^{2+} gradient, occupancy of the calcium sites in the low-affinity state, and of the ADP and P_i sites, slows down ATP breakdown both by inhibiting the enzyme turnover and by increasing the frequency of reverse cycles. Such an intrinsic enzyme regulation can provide kinetic means for a high degree of energy conservation even in conditions far from equilibrium.

Abbreviations: SR, sarcoplasmic reticulum; Me_2SO, dimethyl sulfoxide

2. MATERIALS AND METHODS

Leaky vesicles reconstituted from purified Ca^{2+}-dependent ATPase were prepared as de-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

scribed by MacLennan et al. [8]. $^{32}P_i$ was purified as described [9]. ATPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP [10]. Synthesis of ATP was determined by measuring [γ-^{32}P]ATP formed from ADP and $^{32}P_i$, the excess of $^{32}P_i$ being extracted from the assay medium as phosphomolybdate with 2-butanol-benzene as described [9,10]. The total ATP content in the medium was assayed enzymatically using hexokinase and glucose-6-phosphate dehydrogenase as in [11].

3. RESULTS

In all experiments described here we used purified SR ATPase [8], reassembled in 'leaky' vesicles. Utilization of ATP by the preparation is not accompanied by net accumulation of Ca^{2+}. Therefore, a transmembrane Ca^{2+} gradient does not contribute to equilibration of the reaction. It is shown in fig.1 that mM Ca^{2+} inhibits the rate of [γ-^{32}P]ATP hydrolysis by SR ATPase in the presence of ADP and P_i concentrations which are far from equilibrium. This inhibition that is due to calcium occupancy of the transport sites in the low-affinity state is also observed in the presence of Me_2SO which is known [12,13] to inhibit the hydrolytic reaction by a different mechanism (see below). In parallel with the inhibition of ATP hydrolysis, an increase in the rate of [γ-^{32}P]ATP synthesis is noted as the Ca^{2+} concentration is increased up to the millimolar range (fig.1). Such an increase of [γ-^{32}P]ATP synthesis is much more pronounced when Me_2SO is present. In fact, the ratio between ATP hydrolysis and ATP synthesis decreases from 55 to 7 in totally aqueous media, and from 10 to approx. 1 in the presence of Me_2SO, as the concentration of Ca^{2+} is increased from 0.5 to 4.0 mM (fig.1, inset).

In the presence of a Ca^{2+} concentration sufficiently high (3 mM) to inhibit ATP hydrolysis, an increase of the P_i concentration from 0.2 to 1.0 mM does not significantly change the residual rate of [γ-^{32}P]ATP hydrolysis and increases slightly [γ-^{32}P]ATP synthesis (fig.2A). The effect of P_i is much greater in media containing Me_2SO (fig.2B); in this case, a high stimulation of [γ-^{32}P]ATP synthesis, as well as a large inhibition of [γ-^{32}P]ATP hydrolysis, are noted. This is in agreement with the known ability of Me_2SO to reduce the P_i concentration required to form

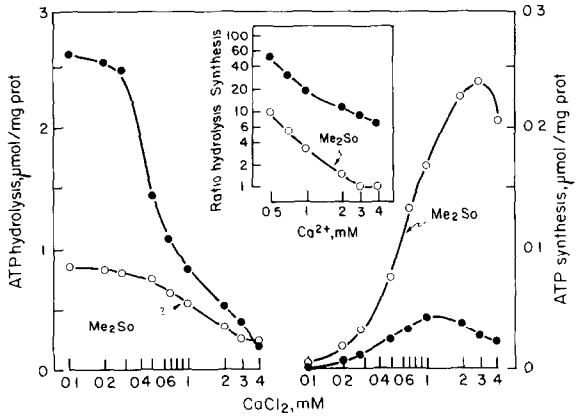

Fig.1. Me_2SO and Ca^{2+} effects. The reaction media contained 30 mM Tris-maleate buffer (pH 7.0), 5 mM $MgCl_2$, 2 mM P_i, 0.1 mM ATP, 0.1 mM ADP without cosolvent (●) or with 20% (v/v) Me_2SO (○). The reaction was performed at 37°C, started by the addition of leaky vesicles to 25 μg protein/ml, and arrested after 1 min (●) or 3 min (○) by addition of trichloroacetic acid to a final concentration of 10% (w/v). For ATPase activity (left), [γ-^{32}P]ATP and nonradioactive ATP were used. For ATP synthesis (right), $^{32}P_i$ and nonradioactive ATP were used.

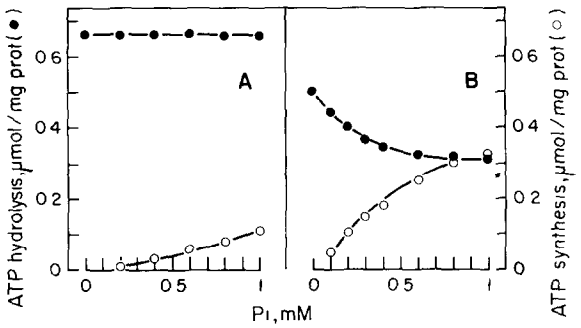

Fig.2. P_i dependence. The reaction media contained 30 mM Tris-maleate buffer (pH 7.0), 5 mM $MgCl_2$, 3 mM $CaCl_2$, 40 μM ATP, 60 μM ADP without cosolvent (A) or with 20% (v/v) Me_2SO (B). In both (A) and (B), the reactions were started by the addition of leaky vesicles to 15 μg protein/ml and arrested after 3 min at 37°C by addition of trichloroacetic acid to a final concentration of 10% (w/v). For ATPase activity (●), [γ-^{32}P]ATP and nonradioactive P_i were used. For ATP synthesis (○), nonradioactive ATP and $^{32}P_i$ were used.

phosphorylated ATPase intermediate by reversal of the hydrolytic reaction [12,13]. Therefore, the effects shown in fig.2B are due to P_i (and ADP) occupancy of the catalytic site.

The experiments shown in figs 1 and 2 were carried out by measuring $^{32}P_i$ production in the reaction started in the presence of [γ-^{32}P]ATP and non-radioactive P_i, or [γ-^{32}P]ATP production in reactions started in the presence of ATP and $^{32}P_i$. In this manner the frequency of forward and reverse cycles to the overall enzyme activity was assessed separately. However, a precise determination of the ratio of these activities was prevented by isotope exchange and therefore, progressive change in specific radioactivity of the two substrate pools. This interference was more evident when the reaction rates were higher, as in totally aqueous media, or with higher protein concentrations even in the presence of Me_2SO, thereby producing apparently non-linear activities following prolonged incubations (fig.3A). We then proceeded to monitor total ATP, by coupled enzyme assays, and found a time-dependent reduction in all conditions studied (fig.3B). This indicates that, as expected, the reaction was far from equilibrium, and the enzyme turnover was constant in each experimental condition (fig.3B), although much slower in the presence than in the absence of Me_2SO. Furthermore, determination of net ATP breakdown corrected by [γ-^{32}P]ATP synthesis, as in

$$\frac{V_{hyd}}{V_{synth}} = \frac{V_{hyd}^{net} + V_{synth}}{V_{synth}}$$

gave us a ratio of 1.35 between the frequency of forward and reverse ATP cycles in the presence of DMSO which could only be approximated by the measurements shown in fig.1.

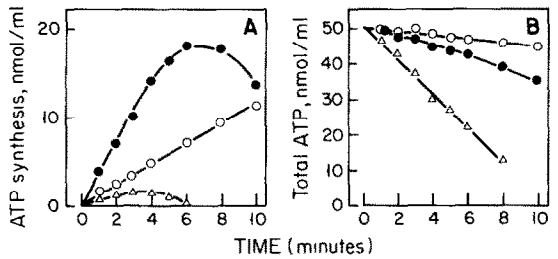

Fig.3. Decrease of total ATP concentration. The reaction media contained 30 mM Tris-maleate buffer (pH 7.0), 5 mM $MgCl_2$, 3 mM $CaCl_2$, 2 mM $^{32}P_i$, 50 μM ATP, 100 μM ADP without cosolvent (△) or with 20% (v/v) Me_2SO (○, ●). The reaction was started by the addition of leaky vesicles to 15 μg/ml (△, ○) or 45 μg protein/ml (●). After different incubation intervals the reaction was quenched by heating the reaction mixture at 100°C for 15 s followed by quick cooling in ice. A sample was used to measure the amount of [γ-^{32}P]ATP synthesized (A) and another sample was used to measure enzymatically the total ATP content of the mixture (B), i.e., the ATP synthesized during the reaction plus the nonradioactive ATP added to the assay medium. After removal of protein by centrifugation, a 0.8 ml sample was mixed with 0.2 ml of a mixture containing 30 mM Tris-maleate buffer (pH 7), 30 mM KCl, 25 mM glucose, 50 mM EGTA, 60 mM $MgCl_2$ and 1 mM NADP and 0.02 ml of a mixture containing glucose-6-phosphate dehydrogenase (80 units/ml) and hexokinase (160 units/ml). The change of optical absorbance was followed at 340 nm.

4. DISCUSSION

Inhibition of SR ATPase hydrolytic activity [10,14] and enhancement of ATP-P_i exchange [10] by high [Ca^{2+}] was previously observed (review [4,5]). In all previous studies the ratio between forward and reverse cycles was much in favor of the forward cycles and only small changes in net ATP breakdown were obtained. We show here that by increasing the affinity of the catalytic site for P_i, it is possible to lower the ratio of forward and reverse cycles from values higher than 50 to a value as low as 1.35, under conditions far from equilibrium. This indicates that a very high degree of energy conservation can be obtained by kinetic regulation of enzyme catalysis. In the case illustrated here for the SR ATPase, such a regulation occurs through a change in the steady-state concentration of enzyme intermediate states, which results in alteration of the rate and direction of substrate flow. This change is produced by complexation of ligands (e.g., P_i) to the enzyme, whereby binding energy is utilized to alter internal equilibria among intermediate states independent of the overall equilibrium of the transformation of ATP to ADP and P_i. It is likely that this type of intrinsic regulation of enzyme catalysis plays a physiological role, since the concentration of Ca^{2+} in the SR lumen of the resting muscle, and the cytoplasmic concentration of P_i are within the effective range. In our experiments, an extreme ex-

ample of very high energy conservation was obtained by the use of Me_2SO which is known to increase the affinity of the enzyme for P_i [12,13].

ACKNOWLEDGEMENTS

This investigation was supported in part by FINEP no.43.83.0052, CNPq and National Institutes of Health (HL-27867).

REFERENCES

[1] Hers, H.G. (1976) Annu. Rev. Biochem. 45, 167–189.
[2] Schatzmann, H.J. (1982) in: Membrane Transport of Calcium (Carafoli, E. ed.) pp.41–108, Academic Press, New York.
[3] Cheung, W.Y. (1980) Science 207, 19–27.
[4] De Meis, L. (1981) The Sarcoplasmic Reticulum Transport and Energy Transduction – Transport in the Life Sciences (Bittar, E.E. ed.) vol.2, John Wiley, New York.
[5] De Meis, L. and Inesi, G. (1982) in: Membrane Transport of Calcium (Carafoli, E. ed.) pp.141–186, Academic Press, New York.
[6] Tanford, C. (1981) J. Gen. Physiol. 77, 223–229.
[7] Tanford, C. (1983) Annu. Rev. Biochem. 52, 379–409.
[8] MacLennan, D.H., Seeman, P., Iles, G.H. and Yip, C.C. (1971) J. Biol. Chem. 246, 2702–2710.
[9] De Meis, L. (1984) J. Biol. Chem. 259, 6090–6097.
[10] De Meis, L. and Carvalho, M.G.C. (1974) Biochemistry 13, 5032–5037.
[11] De Meis, L. and Tume, R.K. (1977) Biochemistry 16, 4455–4463.
[12] De Meis, L., Martins, O.B. and Alves, E.W. (1980) Biochemistry 19, 4252–4261.
[13] De Meis, L. and Inesi, G. (1982) J. Biol. Chem. 257, 1289–1294.
[14] Ikemoto, N. (1974) J. Biol. Chem. 249, 649–651.