Journal of Bioenergetics and Biomembranes, Vol. 18, No. 2, 1986

Modulation of the Kinetic Characteristics of the Sarcoplasmic Reticulum ATPase by Membrane Fluidity

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Received September 11, 1985; revised November 22, 1985

Abstract

$(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum has been reconstituted with dipalmitoylphosphatidylcholine, and the activating effect of ATP and Ca^{2+} on this enzyme has been studied at different temperatures. It has been found that two kinetic forms of the enzyme are interconverted at about 31°C, and this is possibly related to a phase change in the phospholipid which is more directly associated with the protein. Above 31°C the enzyme is less dependent on ATP activation at high ATP concentrations but shows positive cooperativity for Ca^{2+} activation. On the other hand, below 31°C, the reconstituted enzyme is more dependent on ATP for activation at high ATP concentrations than the purified ATPase and does not show cooperativity for Ca^{2+} activation.

Key Words: $(Ca^{2+} + Mg^{2+})$-ATPase; sarcoplasmic reticulum; membrane fluidity; enzyme kinetics.

Introduction

It is well known that the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum has a complex dependence on ATP concentration, which does not follow a Michaelis–Menten type of kinetics (Inesi *et al.*, 1967; Yamamoto and Tonomura, 1968; de Meis and de Mello, 1973; The and Hasselback, 1972; Dupont, 1977; Neet and Green, 1977; Taylor and Hattan, 1979; Moller *et al.*, 1980). Although the reason for that is not fully understood, this observation has been explained by postulating an activation effect of

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0145-479X/86/0400-0113$05.00/0

high ATP concentrations on the turnover of the phosphorylated ATPase intermediate (Verjovski-Almeida and Inesi, 1979; Ariki and Boyer, 1980).

On the other hand, it has been shown (Kosk-Kosicka *et al.*, 1983) that the activation effect of high ATP on the membranous enzyme is absent from the enzyme solubilized with the detergent $C_{12}E_8$. This effect is already found after the incorporation of $C_{12}E_8$ into the membrane, without solubilization (McIntosh and Davidson, 1984). Detergents may release kinetic constraints imposed by the membrane structure on the turnover of the enzyme (Kosk-Kosicka *et al.*, 1983). However, an opposite situation would be to put the enzyme in a rigid membrane. This can be achieved by reconstituting the enzyme with fully saturated synthetic phospholipids like DPPC.[3] This has been done and the results presented in this paper show that the dependence of ATPase on high ATP concentration is increased and at the same time the apparent cooperativity for Ca^{2+} is lost.

Materials and Methods

Chemicals

L-α-1,2-Dipalmitoylphosphatidylcholine was obtained from Fluka, Buchs, Switzerland. ATP, phosphoenolpyruvate, lactic dehydrogenase, and pyruvate kinase were from Sigma, Poole, Dorset, U.K. All other chemicals were analytical grade from Merck, Darmstadt, F.R.G. and Sigma.

$(Ca^{2+} + Mg^{2+})$-ATPase

Sarcoplasmic reticulum was prepared from rabbit back and leg white muscles according to the method of Nakamura *et al.* (1976). $(Ca^{2+} + Mg^{2+})$-ATPase (ATP phosphohydrolase, E.C. 3.6.1.38m) was purified from these membranes by the method of Warren *et al.* (1974) using 0.4 mg cholate/mg protein. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970) showed that this protein was more than 95% pure by weight. It was associated with approximately 30 molecules of phospholipid per molecule of ATPase. A molecular weight of 115,000 was assumed for this protein (Warren *et al.*, 1974). The ATPase was also purified according to procedure 2 of Meissner *et al.* (1973). This form of the enzyme, associated to microsomal intrinsic lipids, will be called in this paper "purified ATPase."

[3] Abbreviations: DPPC: L-α-1,2-dipalmitoylphosphatidylcholine; T_c: main thermotropic transition temperature of the phospholipid bilayer from gel to liquid crystal.

Reconstitution of the enzyme

The ATPase containing approximately 30 lipids per protein, purified according to Warren *et al.* (1974), was reconstituted with pure DPPC, using the method described by Gómez-Fernández *et al.* (1980). Gas–liquid chromatography of the fatty acid methyl esters showed that at least 96–99% of the lipid was of exogeneous origin.

Analytical Assays

Analysis of the lipid content of the reconstituted samples was achieved by quantification of lipid phosphorus (Bartlett, 1959). Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, or from the ATPase molar absorption (Hardwicke and Green, 1974).

ATPase activity was assayed with a regenerating system including 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 4 units/ml pyruvate kinase, 6 units/ml lactate dehydrogenase, 100 mM triethanolamine hydrochloride, pH 7.0, and variable concentrations of Mg^{2+}-ATPase and $CaCl_2$. Free Ca^{2+} concentration was calculated at each temperature, from total Ca^{2+} and EGTA by computations (Fabiato and Fabiato, 1978, 1979) taking into account pH, and the concentration of Mg^{2+}, P_i, K^+, phosphoenolpyruvate, and nucleotides. Control experiments were carried out and it was proved that the increase in concentrations of the auxiliary enzymes did not affect the observed rates. In other control experiments, inorganic phosphate was measured and the rate calculated was in close agreement with that deduced from NADH oxidation through the coupled system.

Results

Modulation of Enzyme Kinetics by Mg^{2+}-ATP

The enzymatic activity of the (Ca^{2+} + Mg^{2+})-ATPase reconstituted with DPPC was measured at different temperatures, the concentration of ATP varying at each temperature between 0.5 μM and 5 mM. The results of two representative temperatures, 24 and 44°C, i.e., below and above T_c of pure DPPC which is 41°C, are shown in the form of Eadie–Hofstee plots (Fig. 1). As can be seen, there is a remarkable increase in specific activity at 44°C with respect to 24°C. The shape of both plots is clearly seen to be totally different, the plot obtained at low temperature being very unusual.

The kinetic data for nucleotide hydrolysis were computed-fitted by a nonlinear regression method to the following steady-state velocity equation (Dupont, 1977; Taylor and Hattan, 1979; Anderson and Murphy, 1983):

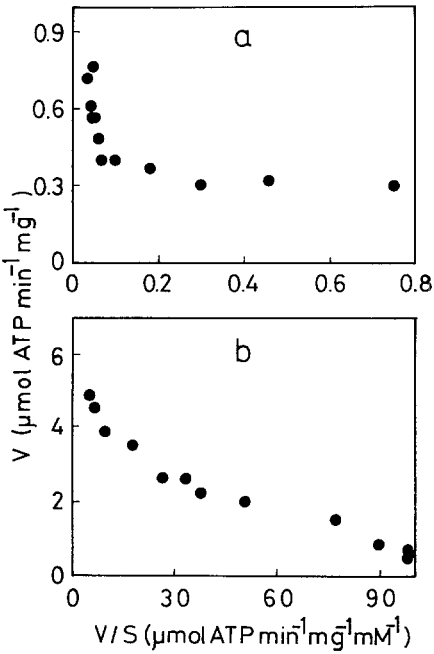

Fig. 1. Eadie–Hofstee plots of the activity of DPPC-reconstituted ATPase at 24°C (a) and 44°C (b). The DPPC/ATPase molar ratio was 146:1.

$$v = \frac{V_1(S/K_m) + V_2(S^2/K_mK_r)}{1 + S/K_m + S^2/K_mK_r}$$

where V_1 and V_2 are the maximal velocities without and with regulatory site occupancy and K_m and K_r are the apparent Michaelis and regulatory site constants, respectively.

Figure 2 shows that two forms of the enzyme are seen with a transition between them that for K_r represents a change from about 0.7 to 0.1 mM (Fig. 2a) whereas K_m increases from about 4 to 14 μM (Fig. 2b). At temperatures above 31°C both K_m and K_r change only slightly.

Data corresponding to purified ATPase, i.e., associated to sarcoplasmic reticulum endogenous lipids, are included as a control. It can be seen that neither K_r (Fig. 2a) nor K_m (Fig. 2b) change appreciably between 24 and 44°C, and since these lipids have no phase transition in this range of temperatures, it is confirmed that the changes observed for the reconstituted ATPase are due to changes in the fluidity of DPPC. It is noteworthy that whereas for purified ATPase K_r values are similar to those of DPPC-ATPase above 31°C, K_m values for purified ATPase are similar to those of DPPC-ATPase below 31°C.

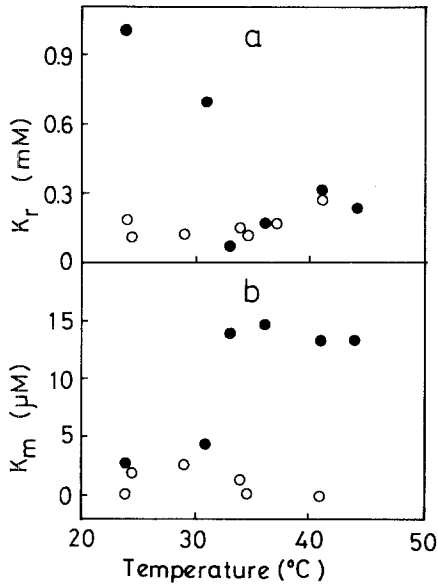

Fig. 2. Variation of apparent K_r (a) and K_m (b) with temperature. K_r and K_m values were deduced from computer-fitting of data as those of Fig. 1 to the equation given in the text. Data obtained with reconstituted ATPase (●) and with purified ATPase (○) are shown.

Modulation of Enzyme Kinetics by Ca^{2+}

These vesicles do not accumulate Ca^{2+} and therefore back inhibition by high Ca^{2+} concentration which is transported inside the vesicles is not expected. It is therefore possible to obtain constant rates of ATP hydrolysis and demonstrate a Ca^{2+} requirement for activation of the enzyme.

High-affinity calcium binding to sarcoplasmic reticulum ATPase occurs with a stoichiometric ratio of 2 with respect to sites phosphorylated with ATP (Yamamoto and Tonomura, 1967, 1968; Makinose, 1969) in steady-state conditions. A positive cooperativity (Hill coeficient about 1.8 to 2) has been found both with fragmented sarcoplasmic reticulum (The and Hasselback, 1972; Makinose, 1969) and with purified ATPase (MacLennan *et al.*, 1972).

The enzymatic activity of ATPase reconstituted with DPPC was measured at different Ca^{2+}-free concentrations, at varying temperatures. Figure 3 shows the results of these experiments at two representative temperatures, 20 and 42°C, i.e., above and below T_c of the pure DPPC. The data are also plotted in this figure according to the Hill formalism. By a computer fitting to Hill plots, n_H values were calculated for the different temperatures. A plot of n_H versus temperature is shown in Fig. 4. Again, as in the case of the ATP modulation, two forms of the enzyme are found, with a transition between

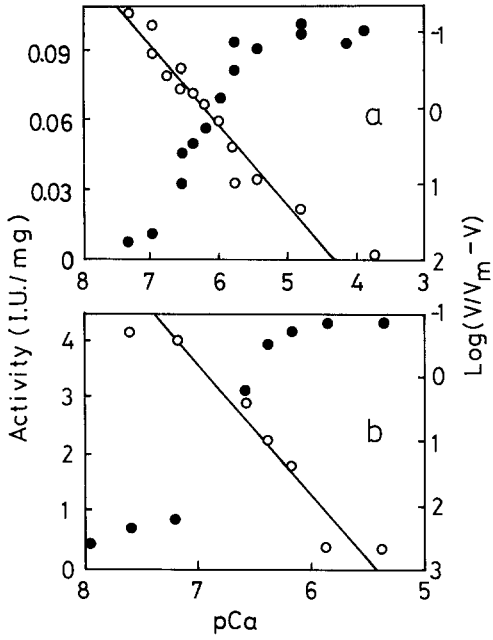

Fig. 3. Changes in ATPase activity with respect to Ca^{2+} concentration (●) and Hill plot of the data (○), of reconstituted enzyme, at 20°C (a) and 42°C (b). The DPPC/ATPase molar ratio was 146:1.

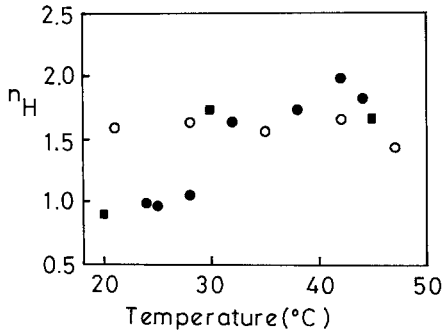

Fig. 4. The dependence of n_H with temperature for purified ATPase (○), and DPPC/ATPase with molar ratios 126:1 (■) and 146:1 (●). n_H values were deduced by computer calculation from data as those of Fig. 3.

them at about 30°C. Below this critical temperature n_H values are around 1, i.e., there is no cooperativity. Above 30°C, n_H oscillates between 1.9 and 1.6, indicating positive cooperativity. Results for two different reconstituted samples are included. The results obtained for ATPase purified according to Meissner *et al.* (1973) are also included as a control, showing that n_H does not appreciably change for this ATPase associated with sarcoplasmic reticulum intrinsic lipids, between 20 and 47°C. This sample has no phase transition in this interval of temperature. Values around 1.6 were found for n_H. These values are similar to those found by other authors using native ATPase (Makinose, 1969).

Discussion

Previous studies done with the $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted with DPPC have shown that the enzymatic activity is remarkably increased at temperatures above 27–32°C (Hesketh *et al.*, 1976) or about 30°C (Higashi and Kirino, 1983). Breaks in Arrhenius plots at about 30°C and about 38°C were reported (Hesketh *et al.*, 1976) and our results closely agree with this pattern (not shown). The break at about 38°C is generally attributed to the melting of bulk lipid, but for the lower one (near 30°C) different explanations have been given. It might be related to the melting of patches of high protein-to-lipid ratio existing at temperatures below T_c (Gómez-Fernández *et al.*, 1980; Hoffmann *et al.*, 1980) or to the melting of the phospholipid annulus (Hesketh *et al.*, 1976). In any case, when the enzyme is associated with rigid DPPC it might be restricted in its rotational mobility and Triton X-100 may set the enzyme free from this restriction (Hidalgo *et al.*, 1978).

Our results further indicate that for ATPase reconstituted with DPPC, an enzymatic interconversion takes place at about 30–31°C, probably due to melting of the phospholipids most directly associated with the protein aggregates. When the interconversion occurs, the temperature being increased, K_r decreases and K_m increases.

The decrease in K_r means that the enzyme associated with fluid phospholipids becomes similar to the purified ATPase, associated with unsaturated phospholipids and hence fluid, as well. This decrease in K_r indicates that dependence of the enzyme on activating ATP is reinforced at temperatures below the observed transition.

As stated above, this is just the opposite of what was found for this enzyme solubilized with $C_{12}E_8$ (Kosk-Kosicka *et al.*, 1983) where it was concluded that the detergent released the kinetic constraints imposed by the membrane structure on the turnover of the phosphoenzyme intermediate. It is interesting to note that it has been reported before (Nakamura *et al.*, 1976;

Hidalgo *et al.*, 1976) that the association of the (Ca^{2+} + Mg^{2+})-ATPase with rigid phospholipids restricted the catalytic cycle, mainly in the step of decomposition of the phosphoenzyme. The observation made in the experiment of Fig. 2a showing that the value of K_r above 31°C of the reconstituted ATPase is similar to that of the purified ATPase corroborates this reasoning.

On the other hand, K_m increases when melting occurs. This possibly means that ATP has a better accessibility to the catalytic centre of the enzyme when it is surrounded by rigid phospholipids than when it is associated with fluid phospholipids. Some tentative explanations for the above now follow. It is possible, for example, that during melting there is a vertical displacement of the enzyme in the membrane of the type described by Borochov *et al.* (1979), the active center thus changing its location. Another explanation might be that there is a decrease in the thickness of the membrane when it is in a rigid state (Ruocco and Shipley, 1982), the catalytic site therefore becoming more exposed to the aqueous environment. Neither should a conformational change of the protein during melting be excluded. It is more surprising that K_m of the purified ATPase, associated with the unsaturated native phospholipids of sarcoplasmic reticulum which are fluid in the whole range of temperatures studied here, is more similar to the reconstituted ATPase surrounded by rigid DPPC than to the form associated with fluid DPPC (Fig. 2b). We have not found a clear explanation for this, but it may be due to a change in the location of the active center, being more exposed to the aqueous environment when reconstituted with exogenous lipids than in the natural membrane. A conformational change during the reconstitution process, however, cannot be ruled out.

The results obtained changing Ca^{2+} concentration shows that two enzymatic forms with transformation of about 30°C are also clearly distinguishable. In this case, the enzyme loses its apparent positive cooperativity when associated with rigid phospholipids. Comparison with fully solubilized ATPase is difficult due to conflicting reports on the Ca^{2+} cooperativity of this solubilized form of the enzyme (Murphy *et al.*, 1982; Watanabe and Inesi, 1982), although recent evidence (Neet and Green, 1977) seems to confirm that there is at least a partial loss of cooperativity due to solubilization by $C_{12}E_8$.

The positive cooperativity of the enzyme for Ca^{2+} probably arises from a conformational change of the enzyme after binding the first Ca^{2+} and, as a result, its affinity for a second Ca^{2+} is increased (Watanabe and Inesi, 1982). This conformational change is apparently not possible when the enzyme is associated with rigid lipids.

In conclusion the association of the (Ca^{2+} + Mg^{2+})-ATPase with DPPC allows the differentiation of two enzymatic forms. The form present at temperatures where the lipid is rigid, behaves as a case opposite to the detergent-solubilized ATPase with respect to its ATP dependency for

activation at high ATP concentrations. On the other hand, the form associated with fluid phospholipids is similar to the enzyme associated with native microsomal vesicles with respect to both its dependence on ATP activation at high ATP concentrations and its pattern of activation by Ca^{2+}.

Acknowledgments

This research was supported in part by Grant No. 0278/81 from Comisión Asesora de Investigación Científica y Técnica (Spain). We thank Dr. Fernández-Belda for heplful discussion.

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