Evaluation of H_2O activity in the free or phosphorylated catalytic site of Ca^{2+}-ATPase

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The sarcoplasmic reticulum Ca^{2+}-ATPase catalyses a reversible calcium transport coupled to phosphate transfer between ATP and water. It has been proposed [Biochemistry (1980) 19, 4252–4261] that the reactivity of the acyl-phosphate bond is dependent on the water activity within the catalytic site. We have tested this hypothesis and found that the polarity in the free catalytic site is lower than that of water, a further and large decrease is observed when the enzyme is phosphorylated by P_i. Phosphorylation by ATP indicates that this polarity change is specifically associated with the formation of the ADP-insensitive phosphoenzyme.

Sarcoplasmic reticulum *Ca^{2+}-ATPase* *Energy transduction* *Phosphorylation* *Water* *Fluorescence*

1. INTRODUCTION

In [3,4] it was calculated that the relative solvation energies of reactants and products are the most important factors to determine the free energy of hydrolysis of 'high energy' bonds. Probably because of the complexity of water structure and of solvation process this role of water has nearly always been neglected in studies of energy transducting systems. However, this concept was applied [1,2] to explain the changes in reactivity undergone by the acyl-phosphate intermediate of Ca^{2+}-ATPase during the working cycle of the enzyme.

Two types of phosphorylated intermediates have been previously observed: E~P or 'high-energy' reacting reversibly with ADP to form ATP; and E*–P or 'low-energy' reacting reversibly with water to liberate P_i. Transition between these two forms of phosphoenzyme appears to be modulated by the occupancy of an internal low affinity calcium site. As a 'working hypothesis' [2] it was proposed that the apparent energy contents of the acyl-phosphate bond is dependent on the water activity within the active site and that, in the absence of calcium, the Ca^{2+}-ATPase catalytic site becomes hydrophobic allowing P_i and aspartic acid to react within the site as in the gas phase to form spontaneously the 'low energy' intermediate: E*–P.

We have tested this hypothesis:

(i) by analysing the water activity dependence of the stability constant of the E*–P bond; and
(ii) by measuring the polarity in the catalytic site with a fluorescent derivative of ATP, TNP–ATP.

Abbreviations: Tris, tris(hydroxymethyl)-aminomethane; MES, morpholinoethane-sulphonic acid; EGTA, ethylene-glycol-bis(amino-2 ethylether),*N,N,N',N'*-tetraacetic acid; TNP–ATP, 2',3'-*O*-(2,4,6-trinitrocyclohexadienylidene)–adenosine triphosphate

2. MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared

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from rabbit muscles as in [5]. Synthesis of TNP–adenine nucleotides, optical measurements and binding assays have been described in [6]. Corrected fluorescence spectra of TNP–ATP were measured at 20°C with a SLM 8000 spectrofluorimeter.

Steady state level of phosphorylation obtained with [γ-^{32}P]ATP or $^{32}P_i$ are shown in table 1. Reaction was quenched with 0.25 M $HClO_4$ 30 s after substrate addition, precipitated proteins were washed extensively on Millipore filters for ATP or by repeated centrifugation for P_i. Sensitivity to ADP was assayed by injecting 1 mM ADP in the reaction mixture 5 s before quenching.

3. RESULTS AND DISCUSSION

3.1. *Water activity and phosphorylation by P_i*

As support for the hypothesis in [2], a dramatic increase of the stability of the acyl-phosphate bond in DMSO–water mixtures was reported. This effect was attributed to a decrease of phosphate solvation by the inorganic solvent and thus of energy needed for P_i to enter the allegedly hydrophobic active site. However, this point is questionable: dipolar aprotic solvents like DMSO can interact with ions by ion–dipole interactions and mutual polarization, so that large anions may be better solvated in DMSO than in water [7,8]. Furthermore, up to a volume fraction of 30%, DMSO is tightly associated with water so that its activity in the mixture is very low (fig.1; [9]). Thus, the major effect of moderate DMSO concentrations in water is to reduce the water activity. The role of water in the phosphorylation process may be better understood by taking explicitly into account the water concentration in the equilibrium:

$$E^* + P_i \rightleftharpoons E^*\text{–}P + n.H_2O$$

$$K_{eq.} = \frac{[E^*\text{–}P]\cdot[H_2O]^n}{[E^*]\cdot[P_i]} = K_{P_i}^{obs}\cdot[H_2O]^n$$

The observed association constant, $K_{P_i}^{obs}$, is therefore related to $K_{eq.}$ by:

$$\log K_{P_i}^{obs} = \log K_{eq.} \models n.\log [H_2O]$$

Association constants were measured by following the intrinsic fluorescence change associated with phosphorylation by P_i [10,11]. Fig.1 shows a very good agreement with the experimental data and in-

Table 1

Correlation of TNP–ATP fluorescence intensity with the state of the catalytic site and evaluation of active site polarity

	Exp. conditions (a)	E–P (b) (nmol/mg) – ADP	+	TNP–ATP (c) fluorescence	Polarity (d) (Z scale)	Water activity (e) (DMSO scale)
	No membranes			1	95	1.0
A	EDTA	–		6–7	84	0.40
B	EGTA + Mg^{2+}	–		6–7	84	0.40
C	Ca^{2+} + Mg^{2+}	–		6–7	84	0.40
D	ATP + Ca^{2+} + Mg^{2+}	2.7	0.4	10–11	80	0.30
E	ATP + 10 mM Ca^{2+} + X-537A + Mg^{2+}	2.5	0.4	10–11	80	0.30
F	ATP + Ca^{2+} + X-537A + Mg^{2+}	2.7	2.4	35–40	70	0.00
G	EGTA + P_i + 20 mM Mg^{2+}	2.9	2.8	35	70	0.00

(a) General conditions were: 200 mM MES–Tris (pH 6.2) and where indicated, 1 mM EDTA, 0.5 mM EGTA, 5 mM Mg^{2+}, 50 μM Ca^{2+}, 10 μM ATP, 20 μM X-537A and 10 mM P_i; (E) 10 mM Ca^{2+}; (G) 20 mM Mg^{2+}; temp. 20°C in (A), (B), (C) and (G); 4°C in (D), (E) and (F)

(b) Steady state level of phosphorylation measured as in section 2. Measurements in the presence of 2 μM TNP–ATP led to the same levels of phosphoenzyme

(c) TNP–ATP fluorescence enhancement determined in conditions similar to that described in fig.3. Bound TNP–ATP at saturation was $n = 6.0 \pm 0.5$ nmol/mg as determined either by fluorescence or by filtration with TNP–[γ-^{32}P]ATP. No significant changes were observed after phosphorylation by P_i or by low concentrations of ATP (10 μM). Measurements of binding were made as in [6]

(d) and (e) Polarity and relative water activity of the TNP–ATP binding site, evaluated according to the calibration shown in fig.2

% DMSO (V/V)	a_{H_2O}	a_{DMSO}	$K_{Pi}^{obs.}$ (M^{-1})
0	1.0	0.0	$1.3\ 10^3$
5	-	-	$3.3\ 10^3$
10	0.9	0.003	10^4
15	-	-	$2.5\ 10^4$
20	0.78	0.014	$6.6\ 10^4$
30	0.66	0.033	n.d.
40	0.54	0.072	n.d.

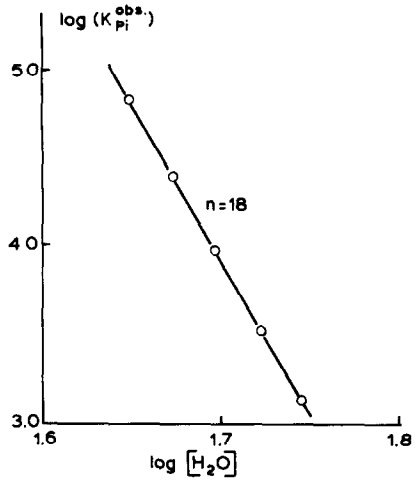


Fig.1. Above: Water and DMSO activities in DMSO–water mixtures calculated from data in [9]. The numbers presented are relative activities expressed as fractions of that of pure water and pure DMSO, respectively. In the same table are compiled association constants for phosphorylation by P_i, measured by intrinsic fluorescence as in [10,11]. Conditions for measurements were: 200 mM MES–Tris, 20 mM Mg^{2+}, 0.5 mM EGTA, 0.11 mg protein/ml (pH 6.1) 20°C and DMSO as indicated. Measurements were not attempted above 20% DMSO mainly because of vesicle aggregation. Below: Evaluation of the number of water molecules released during phosphorylation by P_i. The logarithm of the observed phosphate association constants is expressed as a function of the logarithm of water concentration in various DMSO–water mixtures (0–20% DMSO).

dicates that, to explain the effect of DMSO–water mixtures, phosphorylation by P_i should be accompanied by dissociation of 18 water molecules. This number is far too large to be accounted for by P_i solvation shell only as proposed in [1,2]. It is more likely that most of these water molecules are released from the catalytic site during the reaction. This large number also explains why phosphorylation by P_i produces a large increase in entropy: $\Delta S° = +50$ e.u. (unpublished, [12,13]).

3.2. *TNP–ATP as a reporter of active site polarity*

3.2.1. Spectral properties

Following this, we have attempted to evaluate water activity changes in the Ca^{2+}-ATPase active site using TNP–ATP, a fluorescent ATP analogue recently used to titrate the nucleotide sites of mitochondrial, (Na^+K^+)- and Ca^{2+}-ATPases [6,11,14–16]. TNP–ATP does not phosphorylate Ca^{2+}-ATPase, it is only slowly hydrolysed by the vesicular preparation and its association with the nucleotide sites is very strong ($K_a = 10^7\ M^{-1}$) and specific [6,11,16]. Its fluorescence properties may be used to evaluate the active site polarity since both emission maximum (or transition energy) and

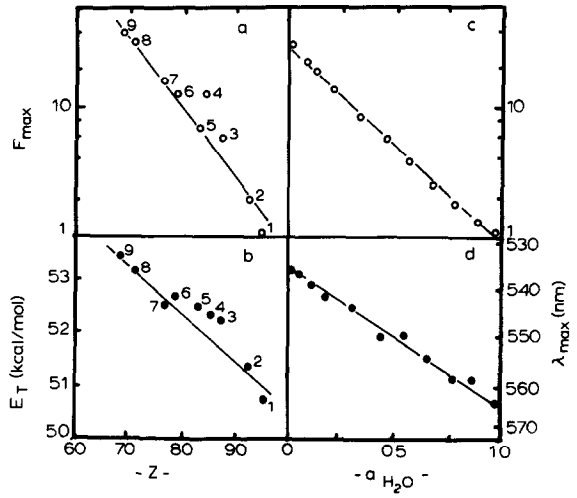


Fig.2. Spectral properties of TNP–ATP in various solvents (a,b) as a function of solvent polarity (Z) as defined in [17], or in DMSO–water mixtures (c,d) as a function of relative water activity as in fig.1. The fluorescence intensities are expressed relative to that measured in pure aqueous buffer (pH 7.5). Maximum emission wavelengths are expressed in nm, or converted in transition energy (E_t (kcal/mol) = $2.86 \times 10^4/\lambda_{max}$ (nm)). As described in [22] the same isomer of TNP–ATP: 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-adenosine triphosphate is found in pure DMSO or in pure water at neutral or alkaline pH. (1) H_2O; (2) 80% H_2O–20% ethanol; (3) 50% H_2O–50% ethanol; (4) ethylene–glycol; (5) methanol; (6) ethanol; (7) 2-propanol; (8) DMSO; (9) DMFA.

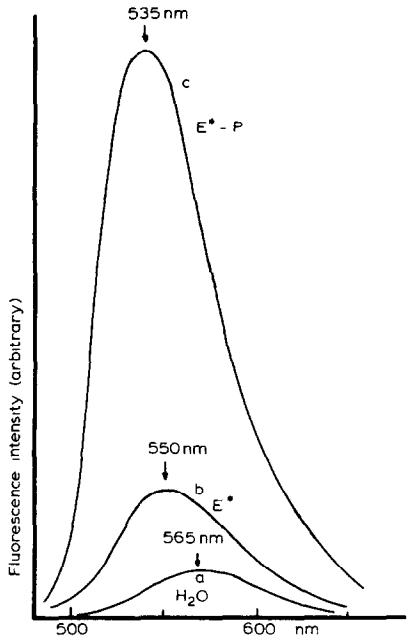


Fig.3. Fluorescence emission spectrum of TNP–ATP in solution or bound to the Ca^{2+}-ATPase, effect of phosphorylation by P_i. Conditions were: 2 μM TNP–ATP, 200 mM MES–Tris, 20 mM Mg^{2+}, 0.5 mM EGTA (pH 6.2) and 20°C. (a) buffer only; (b) buffer and 0.25 mg/ml of Ca^{2+}-ATPase; (c) as in (b) plus 20 mM P_i.

fluorescence intensity show a good correlation with solvent polarity using an empirical polarity scale defined in [17] (fig.2a,b, [18]). A very good correlation was also obtained with water activity in DMSO–water mixtures (fig.2b,c).

Binding of TNP–ATP to Ca^{2+}-ATPase induces a 6–7-fold increase of fluorescence intensity [6,16] together with a blue shift of 15 nm (fig.3), indicating that the polarity of the binding site is significantly lower than that of water, equivalent to that of pure methanol or to a water activity of 40% that of pure water (see table 1). Addition of calcium or magnesium induces no further change in bound TNP–ATP spectral properties in spite of their effects on the tryptophan fluorescence of the protein [19]. This implies that, during the conformation change involved in the $E^* \rightleftharpoons E \cdot Ca$ transition, no appreciable change in the nucleotide site can be detected by TNP–ATP.

3.2.2. Effect of phosphorylation on the fluorescence of bound TNP–ATP

Next, we measured the effect of phosphorylation by P_i or by ATP. These substrates compete with TNP–ATP for binding to the Ca^{2+}-ATPase nucleotide site [6,16] meaning that the free active

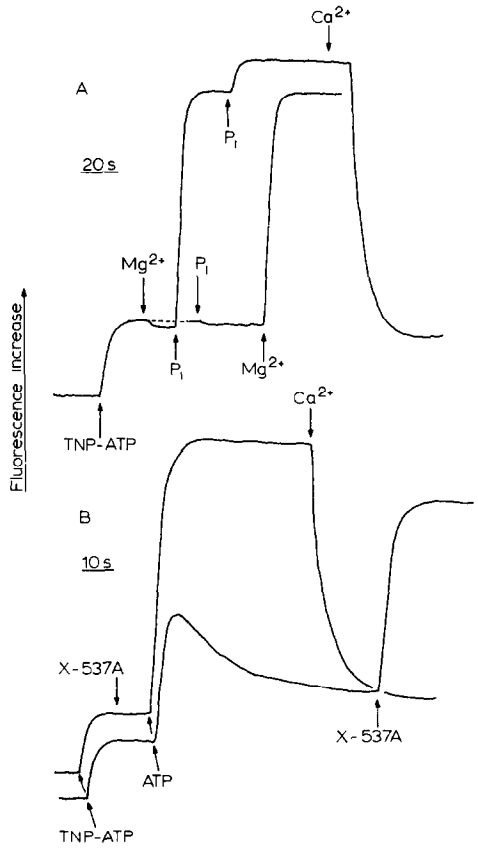
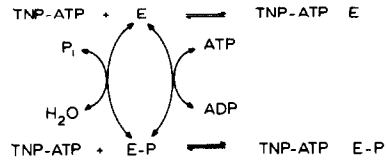


Fig.4. (A) Effect of phosphorylation by P_i on TNP–ATP fluorescence. Conditions were: 200 mM MES–Tris, 0.5 mM EGTA, pH 6.2 and 20°C. Reactants injected: 2 μM TNP–ATP, 20 mM Mg^{2+}, 10 mM P_i. Dephosphorylation was induced by addition of 0.6 mM Ca^{2+} (0.1 mM free calcium). (B) Effect of phosphorylation by ATP on TNP–ATP fluorescence. Conditions were: 200 mM MES–Tris, 100 μM Ca^{2+}, 5 mM Mg^{2+}, pH 6.2 and 4°C to slow down hydrolysis. Reactants injected: 2 μM TNP–ATP, 20 μM X-537A, 10 μM ATP. Addition of 10 mM Ca^{2+} in the presence of X-537A induces the conversion of the ADP-insensitive to ADP-sensitive phosphoenzyme (see table 1). Fluorescence was excited at 405 nm and measured at a right angle, through a Balzers K4 interference filter; protein was 0.11 mg/ml.

site only can be phosphorylated by ATP or P_i as summarized in the scheme below:



We have checked, firstly, that in the presence of a saturating amount of TNP–ATP (2 μM) the Ca^{2+}-ATPase can still be phosphorylated by low concentrations of [γ-^{32}P]ATP or by $^{32}P_i$, secondly that TNP–[γ-^{32}P]ATP was able to bind to the enzyme even after phosphorylation by ATP or P_i (table 1).

Fig.3 and 4A show that phosphorylation by P_i induces a very important increase of TNP–ATP fluorescence. This change is accompanied by a 30–35 nm blue shift (λ_{max} = 535 nm). It indicates that after phosphorylation by P_i, the active site becomes completely free of active water with a polarity equivalent to that found in pure DMSO or even lower (table 1). This is in complete agreement with the observation made above, that a large number of water molecules should be released from the active site during formation of E*–P. Phosphorylation by ATP in the presence of calcium may lead to the formation of a mixture of both E*–P and E~P intermediates [20]. E*–P, the ADP-insensitive intermediate, can be stabilized by preventing calcium accumulation with a calcium ionophore: X-537A [21] (see conditions (F) in table 1). Increasing the external calcium concentration in the presence of X-537A, or allowing calcium accumulation by omitting the ionophore, increases the level of internal calcium so that saturation of the internal low affinity sites produces stabilization of E~P the ADP-sensitive phosphoenzyme ((D) and (E) in table 1).

Fig.4B shows that phosphorylation by ATP induces an important transient increase of TNP–ATP fluorescence after which relaxation to a lower level is observed. Addition of X-537A demonstrates without any doubt that this decreasing phase is due to calcium accumulation and not to ATP exhaustion. The fluorescence of bound TNP–ATP is, therefore, much greater in conditions where the ADP insensitive phosphoenzyme is stabilized. The fluorescence intensity observed in this case is identical to that measured when the enzyme is phosphorylated directly by P_i (see table 1 for correlation). Measurement of the fluorescence drop induced by various concentrations of calcium in the presence of X-537A indicates that reversal of the fluorescence enhancement is induced by calcium binding to a site of low affinity (K_d 2 mM), a value identical to that reported for the internal calcium site [2]. Table 1 indicates that the polarity of the catalytic site around the ADP-sensitive E~P bond should not be significantly different from that of the free site since the small increase in fluorescence observed may well be explained by assuming that 10–15% of the enzyme is in the E*–P form.

4. CONCLUSIONS

These results substantiate the hypothesis in [1,2] that the reactivity of the acyl-phosphate bond is linked to the water activity in the phosphorylated active site and more precisely, that conversion between the high and low energy forms of the phosphorylated intermediates are related to water activity changes in the catalytic site. However, in contradiction with the proposal in [1,2] we have observed that the polarity of the unphosphorylated active site is independent on calcium binding and we think that the property of the Ca^{2+}-ATPase to be phosphorylated by P_i in the absence of calcium gradient is due to a P_i-induced conformation change of the protein, leading to the release of a large number of water molecules from the active site and thus protecting the acyl-phosphate bond from solvation. This transition is probably associated with the intrinsic fluorescence change detected upon phosphorylation by P_i [10,11]. Finally we cannot elude the very puzzling observation that, although only half of the sites titrated by TNP–ATP are able to be phosphorylated (table 1), the spectral property of all bound TNP–ATP was homogeneously affected by phosphorylation. As reported in [6,11], we think that the Ca^{2+}-ATPase enzyme possesses a couple of unequivalent nucleotide binding sites, thus the best explanation we can presently offer is that phosphorylation of one of these two sites induces a concerted transition in both of them.

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