Phosphorylation of Ca^{2+}-ATPase by Inorganic Phosphate in Water-Organic Solvent Media: Dielectric Constant and Solvent Hydrophobicity Contribution†

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The effect of organic solvents on the phosphorylation of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum by inorganic phosphate in the absence of a calcium gradient was investigated. Kinetic analysis of the reaction in water and water-organic solvent media according to a bireactant scheme shows no correlation between changes in kinetic parameters and the dieletric constant of the mixed solvents. The pronounced increase in equilibrium levels of phosphoenzyme in water-solvent mixtures is attributed to changes in the water activity of the medium.

Introduction

The Ca^{2+}-dependent ATPase from sarcoplasmic reticulum can be phosphorylated by inorganic phosphate (Pi) in the absence of a transmembrane calcium gradient [1]. The reaction requires free magnesium, and yields a phosprotein (E ~ P) in which the phosphate is covalently bound to an aspartyl residue of the enzyme [2]. It has been shown [3, 4] that the binding of Pi and Mg^{2+} to the ATPase occurs randomly, and the formation of a ternary complex Mg · E · Pi precedes the phosphorylation reaction (Scheme 1). Also, addition of one ion to the enzyme increases its affinity for the other ion.

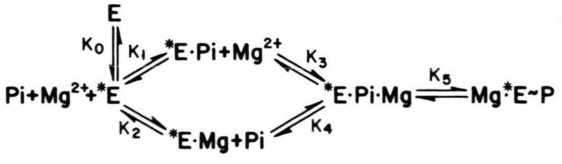
In spite of the "high energy" nature of the acylphosphate bond formed, no energy source for the phosphorylation reaction has yet been identified.

In 1980, de Meis *et al.* [5] proposed that in the absence of a calcium gradient the catalytic site of the enzyme would be hydrophobic, allowing the reaction to occur in a gas phase-like environment. The diminished solvation of the reactants would lead to spontaneous formation of the aspartylphosphate [6], the major thermodynamic barrier for the reaction being the entry of Pi into the hydrophobic site. The authors reported that the additon of organic solvents to the enzyme assay medium stimulated significantly the phosphorylation reaction, besides attenuating or even abolishing the inhibitory effect of high pH [1] and low temperatures [7, 8]. These solvents were also shown to increase the partition coeficient of Pi between organic and aqueous phases.

This cosolvent effect can be ascribed to increases in the hydrophobicity of the system, but modifications in the dielectric constant could also account for – at least – part of it, as the solvents used either decrease (DMFA, glycerol) or do not affect (Me_2SO) the dielectric constant of water.

We have investigated the effect of adding various solvents to the assay medium, including one that increases D(NMFA). Our experimental design also included ethanol, which stimulates other systems [9, 10].

Furthermore, we determined the association constants of Scheme 1, at pH 6.0, in water-organic sol-



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Abbreviations: SR, sarcoplasmic reticulum; Pi, inorganic phosphate; $NaDodSO_4$, sodium dodecyl sulfate; NMFA, N-methylformamide, DMFA, N,N-dimethylformamide; Me_2SO, dimethyl sulfoxide; EGTA, ethylene glycol bis (β-aminoethyl ether)N,N′-tetraacetic acid; Tris, tris (hydroxymethyl)aminomethane; D, dielecric constant; E ~ P, phosphoenzyme.

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vent mixtures, and compared the values with those obtained in isodielectric water media. The results are consistent with increased hydrophobicity of the medium being the main source of the cosolvent effect.

Experimental Section

Sarcoplasmic reticulum vesicles from rabbit white skeletal muscle were prepared as described previously [11], [^{32}Pi]Pi was purified by extraction [12] and stored in dilute HCl solution until used. All reaction media were Na^+ and K^+ free, their composition being described in the figure legends.

EGTA and orthophosphate stock solution were prepared by neutralization of the respective acid forms with Tris. Prior to the addition of the enzyme the pH of the reaction media was adjusted to 6.0 with either Tris or maleic acid.

The reaction was started by additon of SR vesicles and quenched with 2 volumes of ice cold 0.5 M $HClO_4$ plus 4 mM Pi. Equilibrium levels of phosphoenzyme were reached with incubation intervals of 1 min in water, water-DMFA and water-NMFA media, and 5 min in water-glycerol and water-Me_2SO media. The final protein concentration was 0.6 mg/ml in experiments with Me_2SO, and 1 mg/ml with all the other solvent systems.

After quenching the precipitated protein was washed 4 times by centrifugation and resuspension in ice-cold 0.125 M $HClO_4$ plus 2 mM Pi. The protein pellet was dissolved in 0.25 ml of a solution containing 2% $NaDodSO_4$, 0.1 M NaOH, 2% Na_2CO_3 and 1 mM Pi. An aliquot was counted in a liquid scintillation counter, and another aliquot was taken for protein determination [13].

Nonspecific binding of ^{32}Pi was measured in control assays where the enzyme was denatured with 0.5 M $HClO_4$ before addition to the assay medium.

Results

Phosphorylation by Pi on cosolvents at 30 °C

The effects of varying organic solvent concentrations on the equilibrium levels of phosphoenzyme are shown in Figure 1. Data from de Meis *et al.* [5] are used for comparison; as their experiments were performed in slightly different conditions, the results are expressed as percent of control E ~ P levels.

With the exception of ethanol, as the organic solvents substitute increasing amounts of water in the assay medium, the phosphoprotein levels rise, up to a certain limit above which further increase in cosolvent concentration results in an inversion of the effect. The concentration range where the solvents stimulate phosphorylation of the enzyme by Pi depends on the solvent used. The most effective solvent, as already noticed by de Meis *et al.* [5], is Me_2SO. NMFA, a solvent that increases the dielectric constant of water (D) shows essentially the same effects as DMFA and glycerol, which affect D on the opposite way. The enzyme is fairly unstable in water-NMFA mixtures and prolonged incubation intervals result in marked decreases in phosphoenzyme formation (data not shown).

Ethanol does not affect the reaction at concentrations lower than 10% (v/v), beyond which it reduces considerably E ~ P levels. Actually, ethanol behaves

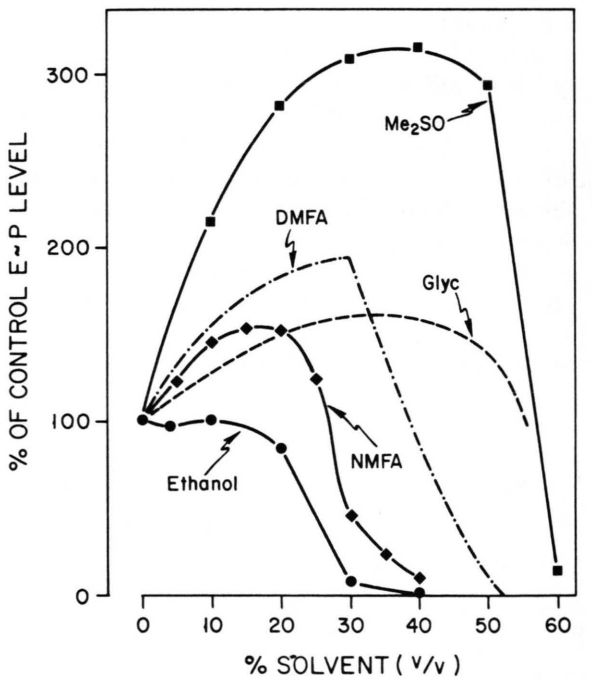

Fig. 1. Equilibrium levels of phosphoenzyme at increasing cosolvent concentration (v/v). The assay medium consisted of 40 mM Tris-maleate buffer (pH 6.0), 1 mM EGTA, 5 mM $MgCl_2$ and 1 mM (^{32}Pi)Pi with NMFA (◆) and Me_2SO (■) and 2 mM (^{32}P)Pi with ethanol (●). Control levels of phosphoenzyme varied from 1.2 to 1.9 μmol E ~ P per g or protein. The reaction was performed at 30 °C as described in methods. DMFA (– · –) and glycerol (– – –) data from de Meis *et al.* [5].

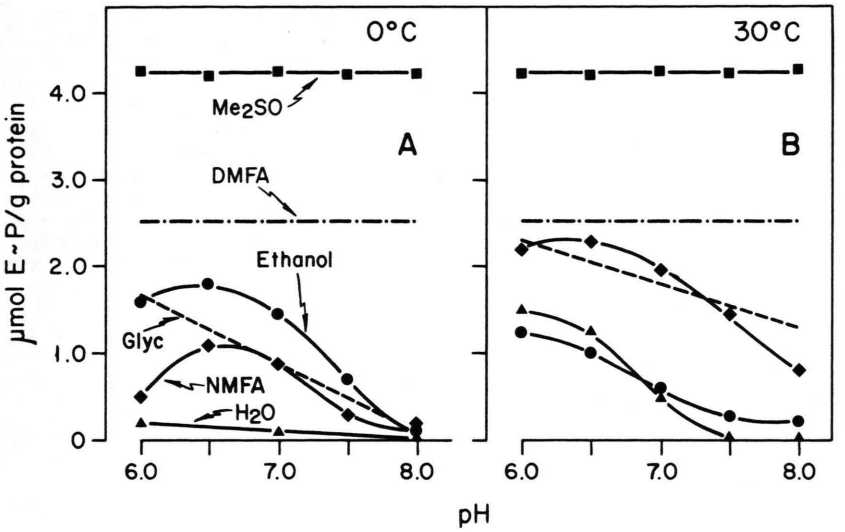

Fig. 2. Effect of pH and cosolvent on equilibrium levels of Phosphoenzyme at 0 and 30 °C. The assay medium consisted of 40 mM Tris-maleate buffer, 1 mM EGTA, 5 mM $MgCl_2$ and 2 mM [^{32}P]Pi without organic solvent (▲) or with 20% ethanol (●); 1 mM [^{32}P]Pi with 20% NMFA (◆) and 40% DMSO (■). The pH of the medium was adjusted to the values in the figure by the addition of Tris or maleic acid. The reaction was performed at 0 °C (A) or 30 °C (B) DMFA (– · –) and glycerol (– – –) data are from de Meis *et al.* [5].

as a noncompetitive inhibitor at this temperature and pH, due to its denaturing properties (data not shown).

Temperature and pH

The phosphorylation of the Ca^{2+}-ATPase by Pi is impaired at 0 °C, and E~P levels increase linearly with temperature up to 20 °C [8]. The pH profile of E~P formation shows a maximum around pH 6.0; rising the assay medium pH results in diminished E~P levels, and at pH 8.0 the enzyme is not phosphorylated by Pi in detectable amounts [1]. In the presence of organic solvents (Fig. 2) both temperature and pH effects are abolished (Me_2SO, DMFA) or less marked (NMFA, glycerol) than in an aqueous medium. Ethanol does reduce the pH effect at 0 °C, displaying a stimulatory effects at pH 6.0 and 7.0; at 30 °C it acts as an inhibitor at pH 6.0, but stimulates the reaction at pH 8.0. These data suggest that at high temperatures the conformation assumed by the enzyme near pH 6.0 is more susceptible to the specific kind of denaturation process induced by ethanol.

Kinetic analysis of the cosolvent effect

In order to assess the effect of the organic solvents on the equilibrium constants of Scheme I, we measured E~P levels at various Pi and Mg^{2+} concentrations. This procedure, used by Punzengruber *et al.* [3] and Martin and Tanford [14], allows the determination of K_1 and K_2 from douple reciprocal plots; K_3 and K_4 are obtained from secondary plots, provided that K_5 or ε (the total number of phosphorylation sites expressed in μmol/g of protein) are known (Fig. 3). In the case of experiments made in water and Me_2SO the K_5 values used are those determined elsewhere [15], leading to an ε value of 5.3 μmol/g of SRV protein. This value corresponds to a stoichiometry of one phosphorylation site per each enzyme chain of mw 120 000, assuming that the ATPase represents 60 to 70% of the total SRV protein in our preparations. The assumption of one site per polypeptide chain was also made in previous reports [3, 14], yielding good results; therefore, we used this same value of $\varepsilon = 5{,}3$ μmol/g of SRV protein to obtain K_3, K_4 and K_5 values in the case of DMFA, NMFA and glycerol. The results are given in Table I. Also listed for comparison are values of the same parameters in water media isodielectric with the water- Me_2SO and water-NMFA mixtures at temperatures lower than 30 °C, as well as those obtained in water at 20 and 30 °C. We cannot make this kind of comparison with the water-glycerol or water-DMFA mixtures, since water reaches D values near 70 at temperatures sufficiently high to preclude exper-

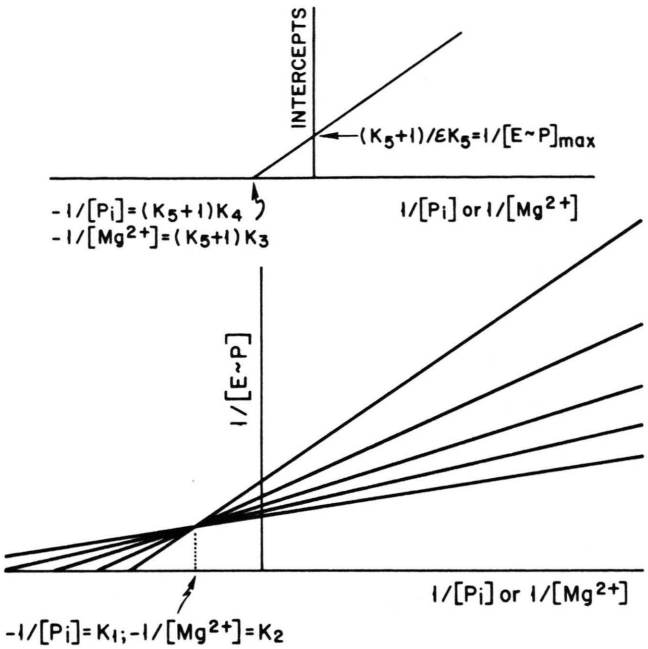

Fig. 3. Graphical determination of the equilibrium constants of Scheme 1 (from Punzengruber *et al.* [3]). Plots of the reciprocal of phosphoenzyme versus reciprocal concentration of one ion, Pi or Mg^{2+} yield lines at different fixed concentration of the other ion, that intersect at points which give values for K_1 and K_2. The inset shows how a replot of the intercepts on the ordinate against reciprocal constant ion concentration yield K_3 and K_4, as well as $E\sim P_{max}$, obtained by extrapolation to Pi $\rightarrow \infty$, $Mg^{2+} \rightarrow \infty$. The Pi concentrations used were 0.25, 0.33, 0.5, 1 and 2.5 mM, and Mg^{2+} concentrations were 0.5, 1; 2.5, 5 and 10 mM, for experiments in water and in binary mixtures of water with DMFA, NNFA and glycerol. In Me_2SO water mixtures the Pi concentrations were 0.1, 0.14, 0.2, 0.4 and 1 mM, Mg^{2+} concentrations being 0.5, 0.7, 1.0, 2.0 and 5.0 mM.

iments, due to extensive thermal denaturation of the ATPase.

In all cases, regardless of the solvent used, the double reciprocal plots were linear as predicted by Scheme I, suggesting that the same reaction model applies to the binary solvent mixtures. No experiments of this kind were conducted in water-ethanol mixtures, due to their denaturing effect on the enzyme at this pH (Figs. 1 and 2).

From Table I, it can be seen that modification of the dielectric constant cannot account for the cosolvent effect. Comparison of the results obtained demonstrated that while all binary mixtures differ widely in their D values, their general effects are similar. Furthermore, when the same parameters are determined in isodielectric water media, different values are found for one or more parameters. Thus, Me_2SO has a marked effect on K_5 and on the maxi-

Table I. Values of association constants and maximum level of phosphoprotein for binary and ternary complex formation from Mg^{2+}, Pi and SR ATPase in water and water-organic solvent mixtures at pH 6.0. In the table C(M) refers to the concentration (molar) of the mixture and D to dieletric constant. Values of K_1 to K_4 are M^{-1} and of $E-P_{max}$, μmol/g protein.

Solvent	C [M]	T [°C]	D[a]	K_1	K_2	K_3	K_4	K_5	$E\sim P_{max}$
Glycerol	4.1	30	67.9	300	180	1480	2040	1.0	3.0
DMFA	3.9	30	70.5	750	160	1130	4720	1.40	3.1
DMSO	5.6	30	73.7	700	430	2200	6400	27.0	5.3
H_2O	–	30	76.7	200	370	440	250	1.70	3.5
DMSO	5.6	20	77.2	400	350	400	710	77.0	4.8
H_2O	–	27.5	77.6	100	80	715	790	1.7	3.2
H_2O	–	20	80.4	100	120	690	700	1.5	3.4
NMFA	3.4	30	87.1	300	120	580	1550	1.5	3.2
H_2O	–	5	86.1	50	60	490	350	1.3	1.5

[a] D values for water, DMFA, DMSO and glycerol solutions from Travers and Douzou [16]; those for NMFA solutions from Rohdewald and Möldner [17].

mal level of phosphoenzyme, while decreasing the synergism of Mg^{2+} and Pi binding. The other solvents seem to act mainly by increasing the secondary association constants, K_3 and K_4, leading to enhanced cooperativity of Mg^{2+} and Pi binding.

Discussion

Modifications in dielectric constant have been assigned an important contribution to the effects of organic solvents in enzyme-catalyzed reactions. However, analysis of the results presented here suggests that if they do have a role an phosphorylation of the SR ATPase by Pi in mixed solvents, it must be a minor one. Attempts to correlate the observed phenomena with the hydrogen bonding capacibility of the cosolvents are not more successful. Me_2SO and DMFA are far more efficient in abolishing pH and temperature effects than the other solvents, and these observations could arise from diminished solvation of Pi, which would lead to increased activity of this anion, but the other solvents, all able to solvate anionic species, display inequivocal activating effects. Thus, it is apparent that the stimulatory properties shared by all the solvents tested can only be explained by a feature common to them all, *i.e.*, increases in hydrophobicity of the medium due to modifications in water microstructure.

As pointed out by Maurel [18], changes in macroscopic values of the dieletric constant are of little relevance, if any, to solvent effects in various enzyme systems, and can be over-shadowed by solvent perturbations of the interaction between water and enzyme or substrate. Another important point [19] is that changes in water concentrations can be important in enzymic reactions where water is a reactant or product.

The fact that ethanol shows a different action profile when compared to other cosolvents does not invalidate the hypothesis of a general hydrophobic effect, because this particular solvent is known to influence protein stability in a complex way [20 – 22].

Finally, the increases in association constants brought about by all the solvents and in K_5 by Me_2SO cannot account wholly for the increased equilibrium levels of phosphoenzyme. It is possible that the organic solvents influence the equilibrium known to exist between two conformations of phosphoenzyme, E (phosphorylated by ATP, but not by Pi) and *E, which reacts with inorganic Pi (Scheme I). In water, in the absence of calcium, the equilibrium favors *E ($K_{eq} = {}^*E/E > 1$). In binary mixtures, in the same conditions, K_{eq} would assume much higher values: *E would be the only stable conformation, and all the ATPase units would be react with Pi: with Me_2SO, levels of phosphoenzyme higher than with the other solvents should be detected due to its pronounced effects on K_5.

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