Role of Water, Hydrogen Ion, and Temperature on the Synthesis of Adenosine Triphosphate by the Sarcoplasmic Reticulum Adenosine Triphosphatase in the Absence of a Calcium Ion Gradient†

Leopoldo de Meis,* Orlando Bonifácio Martins,‡ and Elias Walter Alves‡

ABSTRACT: The aim of this study was to test the hypothesis that in the Ca^{2+}-ATPase of sarcoplasmic reticulum the conversion of the "low-energy" and "high-energy" phosphoenzyme is promoted by the entry of water into the catalytic site of the enzyme. For this purpose, the effect of the organic solvents dimethyl sulfoxide, *N*,*N*-dimethylformamide, and glycerol on the phosphorylation by P_i and on ATP synthesis by the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum was studied in the absence of a transmembrane Ca^{2+} concentration gradient at different pH values and temperatures. In the absence of organic solvents, the concentration of P_i required for half-maximal phosphorylation of the enzyme varied with the pH of the medium, being 1.5 mM at pH 6.0 and too high to measure at pH 8.0. In the presence of organic solvents the apparent K_m for P_i showed little variation with the pH of the medium; in the presence of either 5.4 M glycerol or 3.9 M *N*,*N*-dimethylformamide it was 0.5 mM at pH 6.0 and 0.7–1.5 mM at pH 8.0 and with 5.6 M dimethyl sulfoxide it was 0.007 mM at pH 6.0 and 0.002 mM at pH 8.0. In the absence of organic solvents the phosphorylation of the enzyme by $^{32}P_i$ was impaired as the temperature was decreased from 20 to 0 °C while with organic solvents present phosphoenzyme formation was unchanged between 0 and 37 °C. The time constant of phosphorylation and phosphoenzyme hydrolysis was measured at different pH and temperatures and the equilibrium constant between dephosphoenzyme and phosphoenzyme calculated from these values. In the presence of dimethyl sulfoxide the K_{eq} increased as the temperature was decreased. The ΔH° and ΔS° of phosphoenzyme formation in the presence of dimethyl sulfoxide at pH 6.0 were −21.2 kcal/mol and −65.0 cal/(deg mol), respectively. The partition of P_i from an aqueous phase into a hydrophobic phase increased when glycerol or dimethyl sulfoxide was added to the aqueous phase. Hydrolysis of ATP was reduced by the three organic solvents used irrespective of the medium temperature. In the presence of either dimethyl sulfoxide or *N*,*N*-dimethylformamide this inhibition was not accompanied by a decrease in the steady-state level of phosphoenzyme. In the presence of 5.6 M dimethyl sulfoxide at 0 °C and pH 8.0 and after addition of Ca^{2+}, the phosphoenzyme formed from P_i did not transfer its phosphate to ADP; synthesis of ATP was observed only if the dimethyl sulfoxide concentration was reduced. The Ca^{2+} concentration required for half-maximal synthesis of ATP varied with temperature. At pH 8.0 it was 4×10^{-4} M at 30 °C and decreased at 0 °C to $(1–2) \times 10^{-6}$ M. At 0 °C and pH 8.0 the Ca^{2+} concentration required for half-maximal ATPase activity was $(3–4) \times 10^{-7}$ M. These results are consistent with the hypothesis that water is required for the conversion of the low-energy and high-energy phosphoenzyme.

The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum vesicles can catalyze both the hydrolysis and the synthesis of ATP (Inesi, 1972; Hasselbach, 1978; Tada et al., 1978; de Meis & Vianna, 1979). For the hydrolysis of ATP, the catalytic cycle is initiated by the transfer of the terminal phosphate of ATP to an aspartyl residue of the enzyme, forming an acyl phosphate residue. This reaction is dependent on the binding of Ca^{2+} to high-affinity sites of the enzyme (K_s = 1–3 μM at pH 7.0) located on the outer surface of the vesicle membrane. After phosphorylation of the enzyme, the Ca^{2+} bound to the outer surface of the vesicle is rapidly translocated across the membrane and, because of a marked reduction in the Ca^{2+} binding association constant (K_s = 1–3 mM at pH 7.0), released into the vesicle lumen. Thus, Ca^{2+} is released against a concentration gradient, and the phosphoenzyme subsequently undergoes hydrolytic cleavage.

Ca^{2+} efflux, coupled with the synthesis of ATP, is observed when vesicles previously loaded with a high concentration (>1 mM) of Ca^{2+} are incubated in a medium containing ADP, P_i,[1] and EGTA (Hasselbach, 1978; de Meis & Vianna, 1979). Under these conditions the enzyme is phosphorylated by P_i. This is followed by the binding of Ca^{2+} to low-affinity sites located on the inner surface of the vesicle membrane (Chaloub et al., 1979). After the translocation of Ca^{2+} across the membrane, the phosphoenzyme transfers its phosphate to ADP. EGTA is required in the incubation medium because the binding of Ca^{2+} to the external high-affinity site impairs the phosphorylation of the enzyme by P_i regardless of the Ca^{2+} concentration in the vesicle lumen. The catalytic cycle of the enzyme can be described according to Figure 1 (Carvalho et al., 1976; de Meis & Vianna, 1979).

This sequence includes two distinct functional states of the enzyme, E and *E. The Ca^{2+} binding sites in the E form face the outer surface of the vesicles and have a high affinity for Ca^{2+}. In the *E form the Ca^{2+} binding sites face the inner surface of the vesicles and have a low affinity for Ca^{2+}. The E form is phosphorylated by NTP but not by P_i, while the *E form is phosphorylated by P_i but not by NTP. Conversion of *E into E occurs very slowly in the absence of NTP.

† From the Centro de Ciências de Saúde, Instituto de Ciências Biomédicas, Departamento de Bioquímica, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21.910, Rio de Janeiro, RJ, Brasil. *Received March 20, 1980.* This investigation was supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil (CNPq), Convênio FUJB-FINEP-B/76/79/082, CEPG-UFRJ, ABIF and PNUD/UNESCO/RLA/024.

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[1] Abbreviations used: NTP, nucleoside 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; Me_2SO (DMSO in the figures), dimethyl sulfoxide; DMFA, *N*,*N*-dimethylformamide; $NaDodSO_4$, sodium dodecyl sulfate.

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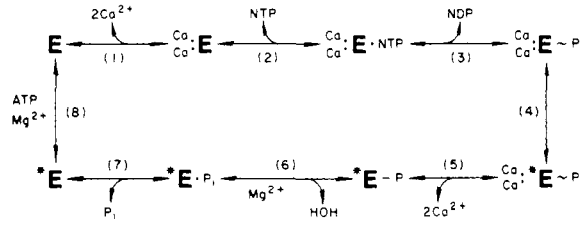

FIGURE 1: Reaction sequence as described in the text.

Table I: ΔH of Hydrolysis in Gas Phase and in Water[a]

reaction	ΔH (kcal/mol) gas phase	soln in water
$H_3P_2O_7^- + H_2O \rightarrow H_3PO_4 + H_2PO_4^-$	−0.93	−7.3
$H_2P_2O_7^{2-} + H_2O \rightarrow 2H_2PO_4^-$	−75.23	−6.8
$CH_3COOPO_3H^- + H_2O \rightarrow CH_3COOH + H_2PO_4^-$	+5.40	−8.6
$CH_3COOPO_3^{2-} + H_2O \rightarrow CH_3COOH + HPO_4^{2-}$	+32.47	

[a] The values shown in the table are from Hayes et al. (1978).

An important finding related to the mechanism of ATP synthesis is that leaky vesicles are phosphorylated by P_i, forming an acyl phosphate residue even in the absence of a transmembrane Ca^{2+} gradient (Masuda & de Meis, 1973; de Meis & Masuda, 1974; Kanazawa, 1975; Knowles & Racker, 1975; Beil et al., 1977; Boyer et al., 1977; Rauch et al., 1977; Punzengruber et al., 1978; Kolassa et al., 1979; Prager et al., 1979). The phosphoenzyme formed in the absence of a Ca^{2+} gradient has been referred to as "low energy" because it does not transfer its phosphate to ADP (de Meis, 1976; Biel et al., 1977; Hasselbach, 1978). However, in the absence of a Ca^{2+} gradient, a single catalytic cycle can be completed if, after phosphorylation by P_i, ADP and a high concentration (>1 mM) of Ca^{2+} are added to the medium (de Meis & Carvalho, 1974; Knowles & Racker, 1975; de Meis & Tume, 1977). This finding indicates that the transformation of the phosphoenzyme from a form which cannot transfer its phosphate to ADP into a form which can transfer, i.e., from "low energy" to "high energy", can only occur when Ca^{2+} binds first to the site of low affinity (reaction 5) and then this site is transformed into a high-affinity site (reaction 4). The source of energy for both the phosphorylation of the enzyme by P_i and for the synthesis of ATP observed in the absence of a transmembrane Ca^{2+} gradient is not known.

Hayes et al. (1978) recently reported theoretical calculations of the hydrolysis energies of high-energy molecules such as acetyl phosphate and pyrophosphate. The values obtained indicate that, although intramolecular effects (opposing resonance and electrostatic repulsions) have a part in determining the energy of hydrolysis, by far the most important factor in determining these energies is the relative solvation energies of reactant and products. In Table I some values of ΔH of hydrolysis calculated by Hayes et al. (1978) for nonsolvated reactants (gas phase) are compared with the values obtained in water. The table shows that acetyl phosphate and pyrophosphate have similar energies of hydrolysis in water but that there is a large difference between the corresponding energies of hydrolysis when the reactants are not solvated.

The present study, based on the observations of Hayes et al. (1978), examines the possibility that in the absence of a Ca^{2+} gradient the catalytic site of the enzyme is hydrophobic so that P_i and the aspartic acid would react as if in a gas phase; the ΔH for the formation of an acyl phosphate residue would be negative, and the reaction would occur spontaneously. In this case, the major thermodynamic barrier would be the partition of the P_i from the assay medium into the catalytic site of the enzyme. Factors facilitating this partition should also facilitate the phosphorylation of the enzyme by P_i. The phosphoenzyme formed would not be able to transfer its phosphate to ADP because of the larger difference of ΔH of hydrolysis of the acyl phosphate and the pyrophosphate residues of ATP (Table I). The binding of Ca^{2+} to the low-affinity site of the enzyme and its conversion into a site of high affinity would permit the entry of water into the catalytic site with subsequent solvation of both acyl phosphate residue and of ADP. As a result, the ΔH values of hydrolysis of the acyl phosphate and of the pyrophosphate residues would become equal and the synthesis of ATP would proceed spontaneously. Experimental conditions which impair the entry of water into the catalytic site should also impair the synthesis of ATP. According to this hypothesis the concept of high-energy and low-energy forms of phosphoenzyme would be related solely to the distribution of water into the catalytic site.

The present report has tested this hypothesis by measuring the phosphorylation of the enzyme by P_i and the synthesis of ATP in the presence of various water–organic solvents mixtures, namely, dimethyl sulfoxide (Me_2SO), glycerol, and *N*,*N*-dimethylformamide (DMFA).

Methods

Leaky vesicles reconstituted from purified Ca^{2+}-dependent ATPase were prepared from sarcoplasmic reticulum vesicles of rabbit skeletal muscle (de Meis & Hasselbach, 1971) as described by MacLennan (MacLennan, 1970; MacLennan et al., 1971).

$^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and was purified by extraction as phosphomolybdate with a mixture of benzene and isobutyl alcohol, reextraction to the aqueous phase with ammonium hydroxide solution, and finally precipitation as the $MgNH_4PO_4$ salt (Kanazawa & Boyer, 1973). The $^{32}P_i$ was stored in dilute HCl solution until used.

The composition of the incubation medium is described in the figure legends. Prior to the addition of the enzyme, the pH of the assay media was adjusted to the desired value with either Tris or maleic acid. For measuring radioactive phosphoenzyme formation from either $^{32}P_i$ or [γ-^{32}P]ATP, the reaction was started by addition of the enzyme and stopped by addition of 2 volumes of an ice-cold 0.25 M perchloric acid solution. The resulting suspension was centrifuged in the cold at 4000*g* for 8 min. The protein pellet was washed 5 times with 4-mL samples of ice-cold 125 mM perchloric acid solution containing 2 mM orthophosphate. The pellet was then dissolved in 0.5 mL of a solution containing 0.1 M NaOH, 2% Na_2CO_3, 1 mM orthophosphate, and 2% $NaDodSO_4$. An aliquot of this solution was used for protein measurement by the method of Lowry et al. (1951), and another aliquot was counted in a liquid scintillation counter. For measurement of nonspecific binding of $^{32}P_i$ or [γ-^{32}P]ATP, control assays were performed in which the enzyme was denaturated by perchloric acid before addition to the assay medium. The values found varied between 0.05 and 0.20 μmol of $^{32}P_i$ per g of protein, and the results of phosphoenzyme formation shown are corrected for this nonspecific binding of $^{32}P_i$ (de Meis & Carvalho, 1976).

ATPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP. After precipitation with perchloric acid (0.15 M) the $^{32}P_i$ was extracted as the phosphomolybdate complex by using a mixture of benzene and isobutyl alcohol (de Meis & Carvalho, 1974). Synthesis of ATP was deter-

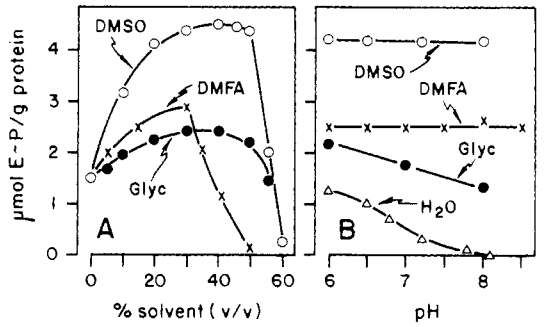

FIGURE 2: Effect of organic solvents and pH on the equilibrium level of phosphoenzyme. (A) The assay medium consisted of 50 mM Tris–maleate buffer (pH 6.0), 10 mM $MgCl_2$, 0.5 mM EGTA, 1 mM $^{32}P_i$, and the concentrations of glycerol (●), DMFA (×), or Me_2SO (○) shown in the figure. The reaction was performed at 36 °C. It was started by the addition of the enzyme to a total of 0.8 mg of protein per mL and stopped after 5 min by the addition of 2 volumes of a cold solution of $HClO_4$ containing 2 mM nonradioactive P_i. (B) The assay medium consisted of 50 mM Tris–maleate buffer, 10 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM $^{32}P_i$ without organic solvent (△) or with 40% v/v glycerol (●), 30% v/v DMFA (×), or 40% (v/v) Me_2SO (○). The pH of the medium was adjusted to the values shown in the figure by the addition of Tris or maleic acid. Other conditions were as in (A).

mined by measuring [γ-^{32}P]ATP formed from $^{32}P_i$. After precipitation of the protein with $HClO_4$ (0.15 M), the $^{32}P_i$ present in the aqueous phase was extracted as a phosphomolybdate complex as described above. The organic phase was discarded, 0.02 mL of 20 mM P_i carrier plus 0.3 mL of acetone were added to the water phase, and this was reextracted with benzene–isobutyl alcohol. This procedure was repeated 3 times. The water phase was counted in a scintillation counter (de Meis & Tume, 1977). ADP free of contaminating ATP (de Meis & Tume, 1977) and [γ-^{32}P]ATP (de Meis, 1972) were prepared as previously described.

Results

Stability of the Enzyme. The enzyme (10 mg of protein/mL) was suspended in solutions containing 30 mM Tris–maleate buffer, 0.2 mM EGTA, 2 mM $MgCl_2$, and either 40% Me_2SO, 40% glycerol, or 30% DMFA at pH 7.0. After different incubation intervals at room temperature, the enzyme suspension was diluted 10-fold in medium of the same composition plus 2 mM $^{32}P_i$ and the amount of phosphoenzyme formed was measured as described under Methods. In agreement with The & Hasselbach (1978), the enzyme was stable in the presence of either Me_2SO or glycerol and essentially the same amounts of phosphoenzyme were obtained after 1 min or 18 h of incubation (data not shown). However, the enzyme was less stable in the presence of DMFA and a 50% decrease in phosphoenzyme formation was observed after 30 min of incubation (data not shown).

Phosphorylation by P_i in the Absence of a Ca^{2+} Gradient. At pH 6.0 the level of phosphoenzyme increased progressively when increasing amounts of organic solvents were substituted for water in the assay medium (Figure 2A). This effect was more pronounced in the presence of Me_2SO. Phosphorylation of the enzyme by P_i was impaired when a large percentage of the water was substituted by the organic solvents. In subsequent experiments, the maximum percentages of water substituted by Me_2SO, glycerol, or DMFA were respectively 40, 40, and 30% (v/v). In these proportions, the final concentrations of solvents were 5.6 M Me_2SO, 5.43 M glycerol, and 3.9 M DMFA. At 25 °C, the dielectric constants of water, Me_2SO, glycerol, and DMFA are respectively 78.4, 46.7, 42.5, and 36.7 (Riddick & Bunger, 1970). The dielectric constant

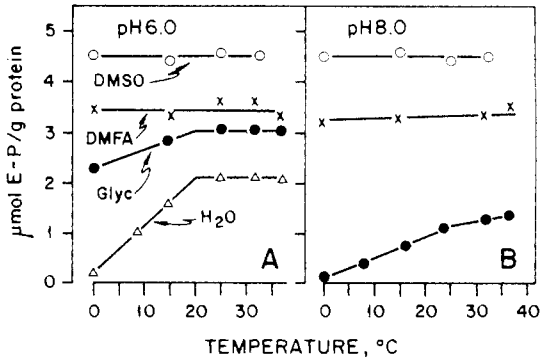

FIGURE 3: Effect of temperature. The assay medium consisted of 50 mM Tris–maleate buffer, pH 6.0 (A) or 8.0 (B), 0.5 mM EGTA, 2 mM $^{32}P_i$, and 10 mM $MgCl_2$ without organic solvents (△) or with 40% glycerol (●), 30% DMFA (×), or 40% Me_2SO (○). The reaction was performed at the temperatures shown in the figure. The reaction time was 10 min. Other conditions were as described in Figure 2A.

Table II: Apparent K_m for P_i at Different pH Values[a]

organic solvent added (v/v)	apparent K_m (M)		
	pH 6.0	pH 7.0	pH 8.0
none	1.5×10^{-3}	10^{-2}	$\gg 10^{-2}$
glycerol (20%)	0.8×10^{-3}		1.5×10^{-3}
glycerol (40%)	0.5×10^{-3}	10^{-3}	10^{-3}
DMFA (13%)	0.5×10^{-3}		1.1×10^{-3}
DMFA (26%)			0.7×10^{-3}
Me_2SO (20%)	7.0×10^{-5}	2.5×10^{-5}	4.0×10^{-5}
Me_2SO (40%)	7.0×10^{-6}	7.0×10^{-6}	2.0×10^{-6}

[a] The apparent K_m was measured as described in Figure 3 by using the concentrations of organic solvents indicated. The $MgCl_2$ concentration was 20 mM without organic solvent and 10 mM under the other conditions.

of a mixture containing 5.6 M Me_2SO is 78.5 and of a mixture containing 5.43 M glycerol is 68.5 (Maurel, 1978).

In previous reports (Masuda & de Meis, 1973; Kanazawa, 1975; de Meis, 1976; Beil et al., 1977; de Meis & Tume, 1977) it was shown that the levels of phosphoenzyme obtained with a given P_i concentration vary with the pH of the medium. In the presence of either Me_2SO or DMFA essentially the same level of phosphoenzyme was attained in the pH range 6.0–8.0, while with glycerol the decrease in phosphoenzyme formation was less marked than with water only (Figure 2B).

Temperature and pH. In agreement with previous reports (Kanazawa, 1975; Masuda & de Meis, 1977), phosphorylation of the enzyme by P_i was reduced at 0 °C and increased linearly with temperature up to 20 °C (Figure 3). In the presence of Me_2SO or DMFA this temperature effect was abolished and phosphoenzyme formation between 0 and 37 °C was essentially unchanged at both pH 6.0 (Figure 3A) and pH 8.0 (Figure 3B). Glycerol does not abolish completely the effect of temperature; at pH 6.0 phosphoenzyme formation at 0 °C is 77% of that at 30 °C, while at pH 8.0 the enzyme is no longer phosphorylated by P_i at 0 °C and formation of phosphoenzyme increases with the temperature up to 37 °C.

Apparent K_m. In previous reports it has been shown that both the initial rate of phosphorylation and the equilibrium level of phosphoenzyme vary with the P_i concentration in the medium. Thus, the apparent K_m for P_i can be estimated by using either one of these parameters in double-reciprocal plots (Masuda & de Meis, 1973; Rauch et al., 1977; Chaloub et al., 1979). In Table II and Figure 4 the apparent K_m for P_i was determined by measuring the equilibrium level of phosphoenzyme. In the absence of organic solvents the concentration of P_i required for half-maximal phosphorylation of the enzyme varies with the pH of the medium (de Meis, 1976; Beil

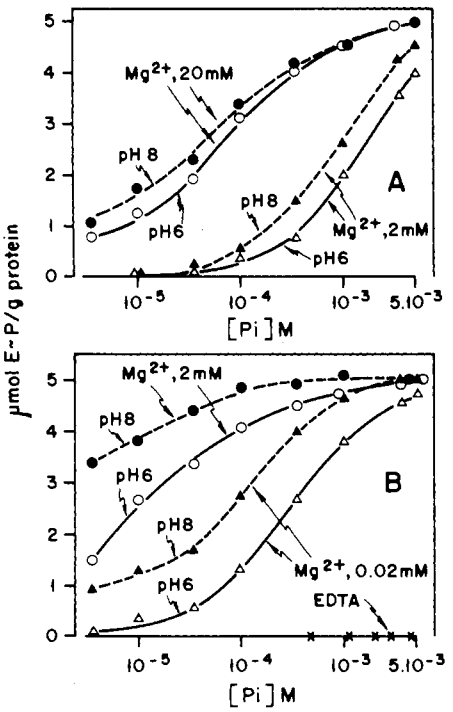

FIGURE 4: P_i and Mg^{2+} dependence in the presence of Me_2SO. (A) The assay medium consisted of 20% (v/v) Me_2SO, 0.1 mM EGTA, 30 mM Tris–maleate buffer, pH 6.0 (○,△) or 8.0 (▲,●), 2 mM (△, ▲) or 20 mM (○, ●) $MgCl_2$, and the concentrations of $^{32}P_i$ shown in the figure. The reaction was performed at 36 °C. Other conditions were as described in Figure 2A. (B) The assay medium consisted of 40% (v/v) Me_2SO, 0.1 mM EGTA, 30 mM Tris–maleate buffer, pH 6.0 (○, △) or 8.0 (●, ▲, ×), and either 1 mM EDTA (×), 0.02 mM $MgCl_2$ (△, ▲), or 2 mM $MgCl_2$ (○, ●). The $^{32}P_i$ concentration was as shown in the figure. The assay was performed at 36 °C. Other conditions were as described in Figure 2A.

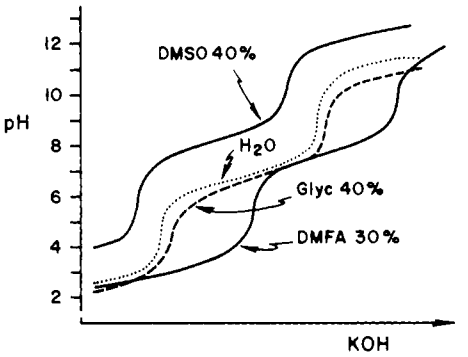

FIGURE 5: Effect of organic solvents on the pK_2 of P_i. H_3PO_4 was dissolved to a final concentration of 50 mM in a solution of 0.08 M HCl without organic solvent (···) or in mixtures (v/v) of either 40% Me_2SO (—), 40% glycerol (- - -), or 30% DMFA (—). Aliquots of 0.01 mL of 5 M KOH were continuously added under intense stirring to 30 mL of each of these mixtures. The pH of the mixture was measured after each addition of KOH. The maximal volume of KOH added was 0.6 mL. The temperature was 25 °C.

Table III: Mg^{2+} Dependence[a]

organic solvent added (v/v)	P_i (mM)	K_{Mg} (mM) pH 6.0	pH 7.0	pH 8.0
none	10.00	2.50	3.0	
glycerol (40%)	5.00	0.40		1.50
DMFA (30%)	2.50			3.50
Me_2SO (20%)	0.01		1.40	
	0.03	2.80	0.91	1.60
	0.10		0.60	
Me_2SO (40%)	0.01	0.60	0.30	0.17
	0.10		0.06	

[a] The assay medium consisted of 30 mM Tris-maleate and 0.1 mM EGTA, and the concentrations of $^{32}P_i$ and organic solvents are indicated. K_{Mg} is the concentration of $MgCl_2$ required for half-maximal phosphorylation. The assay was performed at 36 °C. The reaction was started by the addition of enzyme and arrested after 5 min by the addition of 2 volumes of cold 0.25 M $HClO_4$.

et al., 1977). The values reported are in the range of 1–4 mM at pH 6.0 and 10–25 mM at pH 7.0, while at pH 8.0 the K_m values are so high that they cannot be measured. In the presence of organic solvents the apparent K_m for P_i no longer changes so markedly with the pH of the medium (Table II). The addition of glycerol or DMFA promotes a decrease of the apparent K_m for P_i of 2- to 3-fold at pH 6.0 and of more than 10-fold at pH 8.0. Me_2SO promotes a remarkable decrease of the apparent K_m for P_i (Table II and Figure 4). Substitution of 40% of the water of the assay media by Me_2SO results in a decrease of the apparent K_m for P_i of 200-fold (pH 6.0) to more than 10000-fold (pH 8.0).

Beil et al. (1977) proposed that only primary phosphate ($H_2PO_4^-$) is able to phosphorylate the ATPase in the absence of a Ca^{2+} concentration gradient. Thus, in the absence of organic solvent the different apparent K_m values for P_i measured at pH 6.0 and 7.0 would be related solely to the different concentrations of the ionic species $H_2PO_4^-$ and HPO_4^{2-}. The pK_2 of P_i is 6.7 in both the presence and absence of 40% glycerol (Figure 5); however, only in the absence of glycerol is there a substantial change in the apparent K_m for P_i when the pH is shifted from 6.0 to 8.0 (Table II). In the presence of 30% DMFA or 40% Me_2SO the pK_2 of P_i is 7.5 or 8.1, respectively. In the presence of 20% Me_2SO the pK_2 is 7.5 (data not shown). This finding and the data in Table II indicate that in the presence of organic solvents both $H_2PO_4^-$ and HPO_4^{2-} can phosphorylate the enzyme with nearly the same apparent K_m.

Mg^{2+} Dependence. Mg^{2+} is required for the phosphorylation of the enzyme by P_i in both the presence and absence of organic solvent (Table III and Figure 4). Organic solvents decreased the Mg^{2+} concentration required for half-maximal phosphorylation by P_i. At pH 7.0 and in the presence of 20% Me_2SO (Table III) the Mg^{2+} concentration required for half-maximal phosphorylation decreases from 1.4 to 0.6 mM when the P_i concentration of the medium is raised from 0.01 to 0.10 mM. Figure 4 shows that the apparent K_m for P_i also varied with the Mg^{2+} concentration used.

Equilibrium Constant. The phosphorylation of the enzyme by P_i demonstrates a saturation behavior, indicating the occurrence of a phosphate–enzyme complex previous to the phosphorylation reaction (Masuda & de Meis, 1973; Rauch et al., 1977; Chaloub et al., 1979):

$$E + Mg^{2+} + P_i \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} Mg{\cdot}E{\cdot}P_i \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} Mg{\cdot}E{-}P$$

For an estimation of the K_{eq} (i.e., K_2/K_{-2}) between $Mg{\cdot}E{\cdot}P_i$ and $Mg{\cdot}E{-}P$, the rates of phosphorylation and of phosphoenzyme hydrolysis were measured at different pH and temperatures by using saturating concentrations of P_i and Mg^{2+} (Figures 6 and 7 and Table IV), assuming that $K_1 > K_2$ and $K_{-1} > K_{-2}$ (Kanazawa, 1975; Chaloub et al., 1979). In a previous report it has been shown that the apparent K_m for P_i does not vary with the temperature (Masuda & de Meis, 1977). The rate of phosphoenzyme hydrolysis was measured, maintaining the concentration of $Mg{\cdot}E{\cdot}P_i$ constant, by chasing the decay of radioactive phosphoenzyme after dilution in medium containing nonradioactive P_i.

The values of K_2 and K_{-2} have already been measured at pH 6.0 and in the absence of organic solvent (Rauch et al.,

Table IV: Phosphorylation by P_i. Thermodynamic Parameters[a]

organic solvent added (v/v)	pH	temp (°C)	K_2 (min^{-1})	K_{-2} (min^{-1})	K_{eq} (K_2/K_{-2})	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° [cal/(deg mol)]
none	6.0	25–30	2080–4158	462–594	3.5–9.0	−0.8 to −1.3		
Me_2SO (40%)	6.0	36	20.8	3.5	6.0	−1.1		
	6.0	25	20.8	0.9	23.1	−1.9	−21.2	−65.0
	6.0	0	2.1	0.0039	538.5	−3.4		
Me_2SO (40%)	8.0	36	6.9	2.1	3.3	−0.7		
	8.0	0	0.33	0.0043	76.7	−2.3		

[a] In the absence of organic solvents, the data are from Rauch et al. (1977), Chaloub et al. (1979), and Vieyra et al. (1979). In the presence of Me_2SO, K_2 and K_{-2} were measured as in Figures 6 and 7. Each value represents the average of three experiments. At pH 6.0, there is a linear relationship between log K_{eq} and $1/T$. The equations used are as follows: K_2 or $K_{-2} = 0.693/t_{1/2}$, where $t_{1/2}$ is the time for half-maximal phosphorylation or phosphoenzyme hydrolysis; $\Delta G^\circ = -RT \ln K_{eq}$; $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$; $\log K_{eq_2}/K_{eq_1} = \Delta H^\circ(T_2 - T_1)/(2.303RT_1T_2)$.

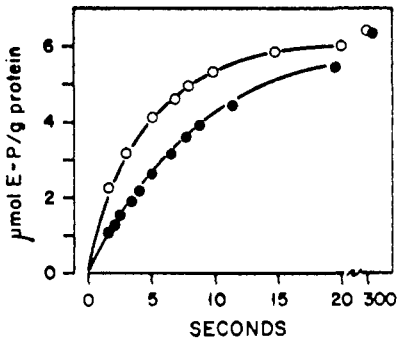

FIGURE 6: Rate of enzyme phosphorylation by P_i in the presence of Me_2SO. The assay medium consisted of 30 mM Tris–maleate buffer, pH 6.0 (○) or 8.0 (●), 0.1 mM EGTA, 1 mM $^{32}P_i$, 10 mM $MgCl_2$, and 40% (v/v) Me_2SO. The temperature was 36 °C. Other conditions were as described in Figure 2A.

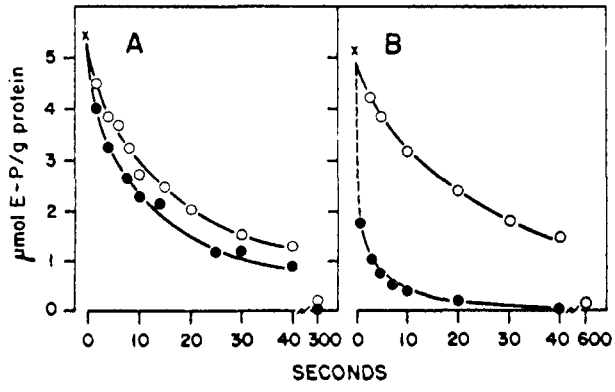

FIGURE 7: Effect of Mg^{2+} and pH on the rate of phosphoenzyme hydrolysis in the presence of Me_2SO. The enzyme (5 mg of protein per mL) was phosphorylated in a medium containing 50 mM Tris–maleate buffer, pH 6.0 (A) or 8.0 (B), 40% (v/v) Me_2SO, 0.5 mM EGTA, 1 mM $^{32}P_i$, and 2 mM $MgCl_2$. After 10 min of incubation, 0.2 mL of this mixture was diluted in 2 mL of medium containing 50 mM Tris–maleate buffer, pH 6.0 (A) or 8.0 (B), 40% (v/v) Me_2SO, 0.5 mM EGTA, 3 mM nonradioactive P_i, and either 2 mM $MgCl_2$ (○) or 15 mM EDTA (●). Both the phosphorylation and the phosphoenzyme hydrolysis were performed at 36 °C. The figure shows the decay of radioactive phosphoenzyme. Other conditions were as described in Figure 2A.

1977; Chaloub et al., 1979; Vieyra et al., 1979) and are in the range of 2080–4158 and of 426–594 min^{-1}, respectively. In the presence of Mg^{2+} and Me_2SO, both the rates of phosphorylation and of phosphoenzyme hydrolysis were markedly decreased compared to these values (Table IV and Figures 6 and 7). When the temperature was reduced from 36 to 0 °C, the decrease in K_2 was less pronounced than that in K_{-2} (Table IV). Therefore, for the formation of phosphoenzyme there was a significant increase in K_{eq}, indicating that in the presence of Me_2SO the phosphoenzyme is more stable at 0 °C than at 36 °C. In contrast, Kanazawa (1975) reported that at pH

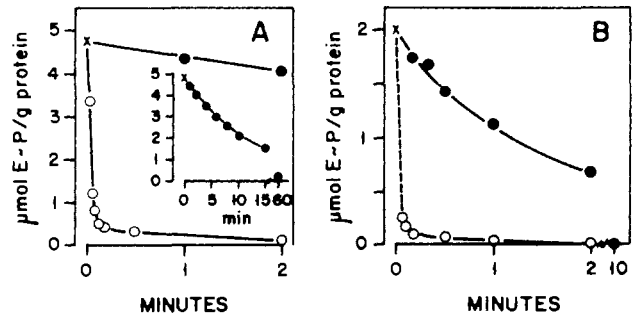

FIGURE 8: Effect of Mg^{2+} on the rate of phosphoenzyme hydrolysis in the absence of Me_2SO. (A) The enzyme (5 mg of protein per mL) was phosphorylated at 0 °C in a medium containing 50 mM Tris–maleate buffer (pH 8.0), 40% (v/v) Me_2SO, 0.5 mM EGTA, 1 mM $^{32}P_i$, and 2 mM $MgCl_2$. After 10 min of incubation, 0.2 mL of this mixture was diluted in 1 mL of medium at 0 °C containing 50 mM Tris–maleate buffer (pH 8.0), 0.5 mM EGTA, 3 mM nonradioactive P_i, and either 2 mM $MgCl_2$ (●) or 15 mM EDTA (○). The reaction was arrested at different incubation intervals after the dilution. Other conditions were as described in Figure 2A. (B) The enzyme (5 mg of protein) was phosphorylated at 30 °C in a medium containing 30 mM Tris–maleate buffer (pH 6.0), 0.5 mM EGTA, 5 mM $^{32}P_i$, and 10 mM $MgCl_2$. After 10 min of incubation, 0.2 mL of this mixture was diluted in 4 mL of a medium at 0 °C containing 50 mM Tris–maleate buffer (pH 8.2), 0.5 mM EGTA, 5 mM nonradioactive P_i, and either 10 mM $MgCl_2$ (●) or 15 mM EDTA (○). The pH of the final mixture was 8.0. The reaction was arrested at different intervals after the dilution. Other conditions were as described in Figure 2A.

7.0, K_{eq} decreases progressively as the temperature of the media is decreased from 37 to 0 °C. This suggests that the slow rate of phosphoenzyme hydrolysis observed at 0 °C (Table IV) is specifically due to the presence of Me_2SO. In the presence of Mg^{2+}, K_{-2} increased from 0.0043 to 0.0866 min^{-1} when the Me_2SO concentration was decreased from 40 to 4% (Figure 8A). The rate of phosphoenzyme hydrolysis was further accelerated when Me_2SO was completely omitted from the assay medium (Figure 8B). This was tested by phosphorylating the enzyme at 30 °C and pH 6.0 and then diluting the radioactive phosphoenzyme in a solution at 0 °C and pH 8.0. Under these conditions K_{-2} was 0.594 min^{-1}.

The ΔH° of the phosphoenzyme formation at pH 6.0 can be calculated from the K_{eq} obtained in the presence of Me_2SO at 36, 25, and 0 °C (Table IV). The value of ΔH° found was −21.2 kcal/mol for the formation of phosphoenzyme which is equivalent to +21.2 kcal/mol for the phosphoenzyme hydrolysis. When compared with the data in Table I, this large positive value of ΔH° for the hydrolysis suggests that in the presence of Me_2SO, P_i and the aspartic acid residue of the catalytic site of the enzyme react as if they were nonsolvated.

The pronounced decrease of the apparent K_m for P_i promoted by Me_2SO (Table II) seems to be mainly due to a change in K_1/K_{-1} (K_s). At 25 °C and pH 6.0 the K_{eq} varies at most sevenfold when Me_2SO is included in the assay media (Table IV). On the other hand, Me_2SO promotes a decrease

Table V: Partition of P_i between Media of Different Hydrophobicity[a]

addn to assay medium (v/v)	organic-aqueous phase partition coeff[b] benzene	75% benzene-25% isobutyl alcohol	55% benzene-45% isobutyl alcohol
none	nd	nd	nd
Me_2SO (40%)	nd	2.0×10^{-5}	1.6×10^{-4}
glycerol (40%)	nd	nd	10^{-5}

[a] The assay medium composition was 50 mM Tris-maleate buffer, 0.5 mM EGTA, 10 mM $MgCl_2$, 2 mM $^{32}P_i$ (4×10^8 cpm/μmol), and the Me_2SO or glycerol concentration shown. To 1 mL of the assay media was added 1 mL of benzene or benzene-isobutyl alcohol. The tube was vigorously stirred for 60 s. After phase separation an aliquot of the benzene-isobutyl alcohol layer was counted in a liquid scintillation counter. With this method, the minimal concentration of P_i which can be detected in the organic phase is 5×10^{-10} M. [b] The partition coefficient was calculated by dividing the concentration of P_i in the organic phase by its concentration in the aqueous phase. nd = nondetectable.

of more than 2 orders of magnitude of the apparent K_m.

Both the ionization of P_i (Figure 5) and the dielectric constant of water are modified to different extents by the organic solvent used. In spite of this variability, the three solvents used tend to decrease the apparent K_m for P_i regardless of the pH of the medium. Therefore, it seems unlikely that the increased affinity of the enzyme for P_i is mediated through changes in electrostatic interactions. A common feature of the three solvents used is to increase the hydrophobicity of the medium. If the catalytic site of the enzyme is hydrophobic, then the partition of P_i from the assay medium into the catalytic site should be facilitated when the difference in hydrophobicity between these two compartments is small. On the other hand, in the absence of organic solvent the hydrophobicity of the catalytic site would vary with the pH of the medium. The partition of P_i between the water phase (phosphorylation medium) and different mixtures of benzene–isobutyl alcohol (organic phase) was measured to test this conclusion (Table V). The hydrophobicity of the organic phase increases with an increase in the benzene/isobutyl alcohol ratio. Isobutyl alcohol is freely soluble in benzene but poorly soluble in water (1:20) and under the conditions described in Table V remained in the organic layer with the benzene while Me_2SO and glycerol remained in the aqueous phase (data not shown). DMFA was not used in this experiment because it distributes into the two layers. P_i only entered in significant amounts into the organic layer when Me_2SO or glycerol was included in the assay medium. The amount of P_i which entered the organic layer increased as the ratio of benzene to isobutyl alcohol decreased. Me_2SO was more effective than glycerol in promoting the entry of P_i into the organic layer.

Stabilization of the Phosphoenzyme by Mg^{2+}. Mg^{2+} had a complex effect on the rate of phosphoenzyme hydrolysis which is dependent on the pH of the medium. At pH 6.0 the rate of phosphoenzyme hydrolysis was slightly faster in the presence of EDTA than in the presence of Mg^{2+}. At pH 8.0, removal of Mg^{2+} from the hydrolysis medium resulted in a sharp acceleration of the rate of phosphoenzyme hydrolysis (Figure 7). This effect of Mg^{2+} was also observed at 0 °C and in the absence of Me_2SO (Figure 8). These findings indicate that in both the presence and absence of Me_2SO the phosphoenzyme containing bound Mg^{2+} is much more stable than the Mg^{2+}-free phosphoenzyme and that the stabilizing effect of Mg^{2+} is abolished when the pH is reduced from 8.0 to 6.0.

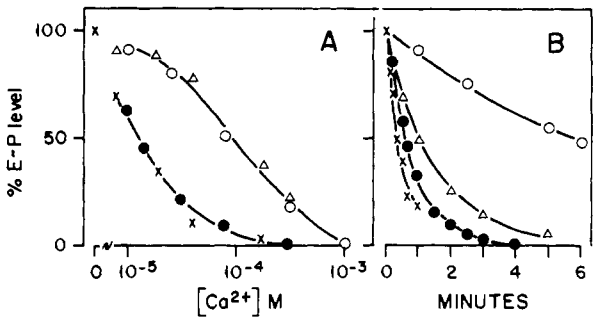

FIGURE 9: Inhibition of phosphoenzyme formation by Ca^{2+}. (A) The assay medium consisted of 30 mM Tris–maleate buffer (pH 6.0), 1 mM $^{32}P_i$, 10 mM (×, ○, △) or 0.2 mM (●) $MgCl_2$, and either 0.5 mM EGTA (zero Ca^{2+}) or the Ca^{2+} concentrations shown in the figure (×) without organic solvent or with (○, ●) 20% (v/v) Me_2SO or (△) 40% (v/v) glycerol. The reaction was performed at 36 °C. It was started by the addition of the enzyme to a total of 0.2 mg of protein per mL and arrested after 15 min by the addition of 2 volumes of cold 0.25 M $HClO_4$ containing 2 mM carrier P_i. (B) The assay medium contained 30 mM Tris–maleate buffer (pH 6.0), 0.1 mM EGTA, 10 mM $MgCl_2$, 1 mM $^{32}P_i$, and either (○) 40% Me_2SO, (●) 20% Me_2SO, (△) 40% glycerol, or (×) 30% DMFA. 15 min after the addition of the enzyme (0.8 mg of protein per mL), Ca^{2+} was added to a final concentration of 2 mM. The reaction was arrested at different incubation intervals after the addition of Ca^{2+}. The reaction was performed at 36 °C.

Inhibition by Ca^{2+}. The K_s of the high-affinity Ca^{2+} binding site of the enzyme varies with the pH of the assay medium (de Meis & Tume, 1977; Verjovski-Almeida & de Meis, 1977). At pH 6.0 it is in the range of 10–20 μM. Figure 9A shows that without organic solvent at pH 6.0 in the presence of 10 mM $MgCl_2$, half-maximal inhibition of the equilibrium level of the phosphoenzyme was attained in the presence of 13 μM Ca^{2+}. In the presence of 40% glycerol or 20% Me_2SO and the same Mg^{2+} concentration, the Ca^{2+} concentration required for half-maximal inhibition increased to 80 μM. When the Mg^{2+} concentration was decreased to 0.2 mM in the presence of 20% Me_2SO, half-maximal inhibition was obtained with 13 μM Ca^{2+}. Essentially the same results were obtained in the presence of 40% Me_2SO (data not shown). These results show that in the presence of organic solvent, Mg^{2+} can prevent the binding of Ca^{2+} to the enzyme. In previous reports (de Meis & Tume, 1977; Rauch et al., 1977; Chaloub et al., 1979) it has been shown that when the decay of the phosphoenzyme is initiated by the addition of Ca^{2+}, the resulting time constant is smaller (0.87–0.35 s^{-1}) than that measured in the absence of Ca^{2+} (8.7 s^{-1}). This indicates that the enzyme form $^*E$ undergoes a slow transition before becoming unreactive to P_i. In the reaction sequence proposed, the cleavage of phosphoenzyme measured in the absence of Ca^{2+} is represented by reactions 6 and 7 (Figure 1) and that in the presence of Ca^{2+} by reactions 6, 7, 8, and 1. A comparison of Figures 7B and 9 shows that the same phenomenon is observed in the presence of organic solvent. At 36 °C in the presence of 40% Me_2SO and Ca^{2+} the phosphoenzyme decayed with a time constant of 0.12 min^{-1} (Figure 9B), while in the absence of Ca^{2+} (Figure 7B) the time constant was 2.08 min^{-1}. In the presence of Ca^{2+}, the rate of phosphoenzyme cleavage varied with the solvent used and its concentration. Figure 9B shows that when the concentration of Me_2SO is decreased from 40 to 20%, the time constant increases from 0.12 to 1 min^{-1}. In the presence of 40% glycerol and 30% DMFA, the time constants of decay were 0.69 and 2.1 min^{-1}, respectively.

ATPase Activity. The & Hasselbach (1977) have shown that Me_2SO inhibits the Ca^{2+}-dependent ATPase activity of sarcoplasmic reticulum vesicles. This is not accompanied by

Table VI: ATPase Activity and Phosphorylation by [γ-^{32}P]ATP[a]

organic solvent added (v/v)	phosphoenzyme (μmol/g of protein)		ATPase act. [μmol of P_i/(g of protein min)]	
	0 °C	36 °C	0 °C	36 °C
none	3.25	2.43	16.00	2950.00
Me_2SO (40%)	2.90	2.90	0	80.00
DMFA (30%)	3.44	3.01	2.71	720.00
glycerol (40%)	1.09	0.58	5.13	560.00

[a] The assay media consisted of 50 mM Tris–maleate buffer (pH 7.5), 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM [γ-^{32}P]ATP, 0.2 mg of enzyme protein per mL, and organic solvents as indicated. When the reaction was performed at 0 °C, the reaction times in the absence and presence of organic solvents were 10 s and 2 min, respectively. At 36 °C, the reaction time was 2 or 10 s.

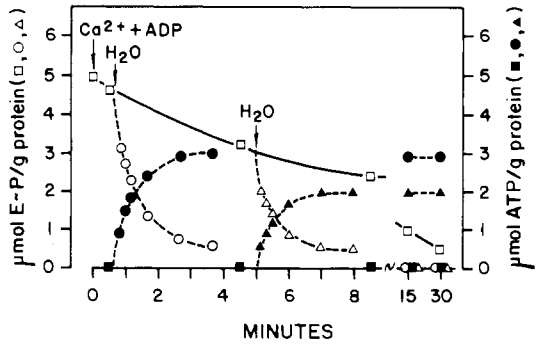

FIGURE 10: Inhibition of ATP synthesis by Me_2SO. The enzyme (7 mg of protein per mL) was phosphorylated at 0 °C in 1.7 mL of a medium containing 30 mM Tris–maleate buffer (pH 8.0), 0.05 mM EGTA, 5 mM $MgCl_2$, 1 mM $^{32}P_i$, and 40% (v/v) Me_2SO; 15 min after the addition of enzyme 0.017 mL of ADP (20 mM) and 0.02 mL of $CaCl_2$ (20 mM) were simultaneously added. The final concentrations of Ca^{2+} and ADP were 0.15 and 0.40 mM, respectively. The reaction was arrested at different incubation intervals after the addition of ADP and Ca^{2+}. In a parallel set of tubes, after the addition of ADP and $CaCl_2$, 0.1 mL of the mixture was diluted in 1.9 mL of a solution of identical composition at 0 °C except that $^{32}P_i$ and Me_2SO were omitted. In the figure this is represented as ($\downarrow H_2O$). Radioactive phosphoenzyme (open symbols) and ATP synthesis (closed symbols) were measured as described under Methods: (□, ■) 40% (v/v) Me_2SO; (○, ●, △, ▲) after dilution of Me_2SO from 40 to 2% (v/v).

a decrease of the steady-state level of the phosphoenzyme formed by [γ-^{32}P]ATP. Comparable results are shown in Table VI, and in addition a similar effect was obtained with the use of DMFA. Glycerol decreased both the ATPase activity and the steady-state level of phosphoenzyme.

Net Synthesis of ATP in the Absence of a Ca^{2+} Gradient. This set of experiments was performed only in the presence of Me_2SO. Figure 10 shows that the synthesis of ATP is abolished in the presence of 40% Me_2SO. After the addition of ADP and Ca^{2+} the phosphoenzyme was slowly hydrolyzed. This was not accompanied by the synthesis of ATP. The inhibition of the ATP synthesis seems to be specifically due to the presence of Me_2SO. A rapid cleavage of the phosphoenzyme coupled with a stoichiometric synthesis of ATP was observed if, after the addition of ADP and Ca^{2+}, the Me_2SO concentration was suddenly decreased from 40 to 2% under conditions where both the temperature and pH were maintained constant. The time constant of both phosphoenzyme disappearance and ATP synthesis was in the range 1.7–1.2 s^{-1}. In all experiments the amount of ATP synthesized never exceeded the number of enzyme sites phosphorylated by P_i. The Me_2SO concentration which inhibited the synthesis of ATP was determined in Figure 11. The concentration of Me_2SO was decreased in the presence of ADP and Ca^{2+}, and both the portion of phosphoenzyme remaining and the amount

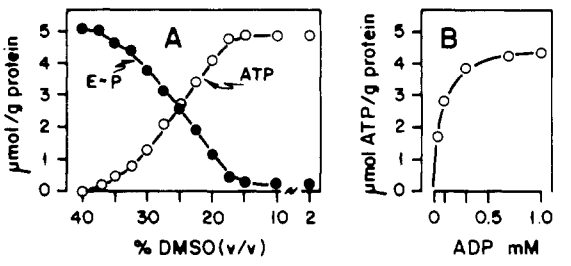

FIGURE 11: (A) Effect of different Me_2SO concentrations on the synthesis of ATP. The enzyme (7 mg of protein per mL) was phosphorylated at 0 °C in a medium containing 30 mM Tris–maleate buffer (pH 8.0), 0.05 mM EGTA, 10 mM $MgCl_2$, 1 mM $^{32}P_i$, and 40% (v/v) Me_2SO. After 15 min, 0.1 mL of this mixture was diluted in 1 mL of a solution at 0 °C containing 30 mM Tris–maleate buffer (pH 8.0), 10 mM $MgCl_2$, 2 mM ADP, 0.1 mM $CaCl_2$, and different concentrations of Me_2SO. 2 min after the dilution the reaction was arrested by the addition of $HClO_4$. Other conditions were as described in Figure 10: (●) phosphoenzyme; (○) ATP synthesis. (B) ADP dependence. The conditions were as in (A) except that Me_2SO was omitted from the dilution medium and the final concentration of ADP was varied as shown in the figure.

of ATP synthesized were measured. This experiment showed that half-maximal synthesis of ATP was reached when the Me_2SO concentration was decreased from 40 to 25%. Under the conditions shown in Figure 10, the apparent K_m of ADP for ATP synthesis was 0.08 mM (Figure 11B). It should be emphasized that the experiments shown in Figures 10 and 11 were performed only at 0 °C and pH 8.0. Under these conditions synthesis of ATP can be measured in the presence of a low Ca^{2+} concentration (0.1 mM).

Ca^{2+} Dependence of ATP Synthesis. In previous reports (de Meis & Tume, 1977; Verjovski-Almeida & de Meis, 1977) it has been shown that at temperatures above 20 °C the binding of Ca^{2+} to the low- and high-affinity sites varies in parallel with the pH of the medium. Thus, in the pH range 6–8, the Ca^{2+} concentration required for half-maximal saturation of the high-affinity site (K_{Ca}^1) is always 1000 times smaller than that required for the low-affinity site (K_{Ca}^2). It has also been shown that K_{Ca}^1 does not vary with temperature (de Meis, 1972). The affinity of these two classes of Ca^{2+} binding sites was indirectly measured either by determining the Ca^{2+} concentration required for half-maximal ATPase activity and half-maximal phosphorylation by [γ-^{32}P]ATP (K_{Ca}^1) or by measuring the Ca^{2+} concentration required for half-maximal synthesis of ATP (K_{Ca}^2) (Figure 12). At 0 °C and without Me_2SO, the values of K_{Ca}^1 found at pH 6.2, 7.0, and 8.0 were respectively 10, 3, and 0.3–0.4 μM. These values are the same as those previously reported at temperatures above 20 °C (de Meis & Tume, 1977; Verjovski-Almeida & de Meis, 1977). A surprising finding was that K_{Ca}^2 does vary with the temperature. At pH 6.2, 7.0, and 8.0 the values found at 30 °C were 20, 3, and 0.4 mM, respectively, and they decreased to 4.5 mM, 0.4 mM, and 0.8–2 μM at 0 °C. Thus, the ratios between K_{Ca}^2 and K_{Ca}^1 are 2000, 1000, and 1000, respectively, at 30 °C and these decrease to 450, 130, and 4–5 at 0 °C. This clearly shows that at 0 °C the difference between the Ca^{2+} affinity for the two binding sites decreases as the pH of the medium is raised and that at pH 8.0 essentially the same Ca^{2+} concentration which activates the hydrolysis of ATP also activates the synthesis of ATP. This peculiar modification of K_{Ca}^2 at 0 °C was not related to the presence of Me_2SO in the medium used to phosphorylate the enzyme by P_i (Figure 13). The enzyme was phosphorylated by P_i at 30 °C and pH 6.0 in the absence of Me_2SO and then diluted 10-fold in ADP-containing medium at 0 °C with varying pH and Ca^{2+} concentrations. A comparison of Figure 13 with

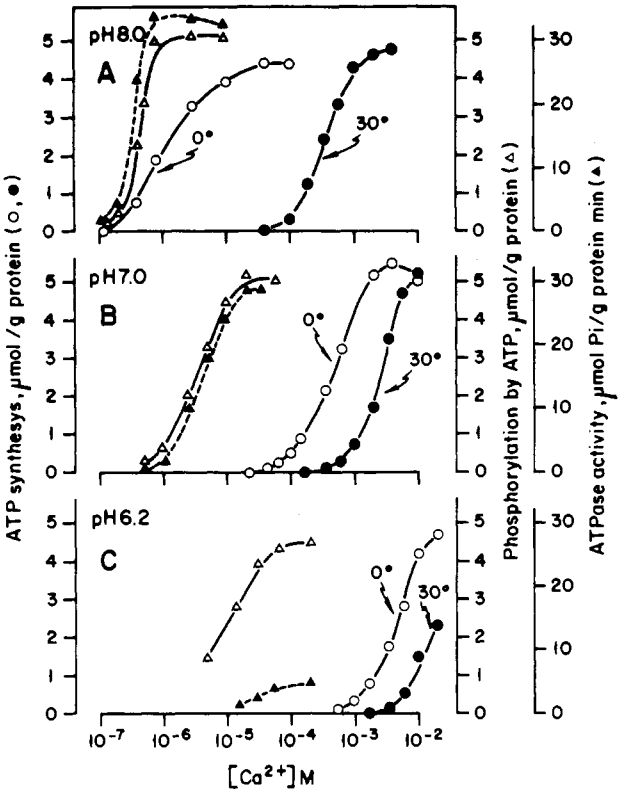

FIGURE 12: Ca^{2+} dependence for the synthesis and hydrolysis of ATP at different temperatures. ATP hydrolysis and enzyme phosphorylation by [γ-^{32}P]ATP (△) were measured at 0 °C in medium containing 60 mM Tris–maleate buffer, 0.1 mM [γ-^{32}P]ATP, 0.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and different EGTA concentrations to yield the free calcium concentrations shown in the figure. The reaction was started by the addition of enzyme, total of 0.8 mg of protein per mL, and arrested after 20 s by the addition of $HClO_4$. Synthesis of ATP was measured at 0 °C (○) and at 30 °C (●). The enzyme was phosphorylated by $^{32}P_i$ in the presence of Me_2SO as described in Figure 11A, at either 0 or 30 °C; 0.1 mL of the phosphoenzyme medium was diluted in 2 mL of a medium at either 0 or 30 °C containing 30 mM Tris–maleate buffer, 1 mM $MgCl_2$, 0.4 mM ADP, and EGTA and $CaCl_2$ concentrations to yield the free calcium concentrations shown in the figure. The reaction was arrested with $HClO_4$ 5 min after the dilution. Phosphoenzyme, ATP synthesis, and ATP hydrolysis were measured as described under Methods. The final pH of the different assay media was either 8.0 (A), 7.0 (B), or 6.2 (C). The Ca^{2+} concentration was calculated by using the association constants for the formation of the Ca·EGTA complex, 2.24×10^4, 2.53×10^2, and 6.44 M^{-1} at pH 8.0, 7.0, and 6.2, respectively.

Figure 12 shows that at each pH, the Ca^{2+} concentrations required for half-maximal synthesis of ATP were the same and therefore independent of the presence of Me_2SO.

Ionophore X-537A. In the experiments described, leaky vesicles reconstituted from purified ATPase were used. Although these vesicles are unable to accumulate Ca^{2+}, the possibility exists that after exposure to Me_2SO they may somehow become sealed. Two experiments were performed in the presence and absence of a large excess (0.4 mM) of the Ca^{2+} ionophore X-537A in order to ensure that the synthesis of ATP measured in Figures 11 and 12 occurred in the absence of a Ca^{2+} gradient across the membrane. The other conditions were as described in Figure 10. In both experiments the number of enzyme sites phosphorylated by P_i and the amount of ATP synthesized in the presence and absence of X-537A were identical (data not shown).

Discussion

The proposed hypothesis states that high- and low-energy forms of the phosphoenzyme in the sarcoplasmic reticulum

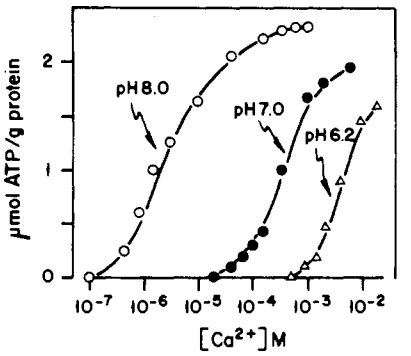

FIGURE 13: Ca^{2+} dependence for ATP synthesis in the absence of Me_2SO. The enzyme (3.2 mg of protein per mL) was phosphorylated in a medium containing 30 mM Tris–maleate buffer (pH 6.2), 10 mM $MgCl_2$, 0.5 mM EGTA, and 4 mM $^{32}P_i$. 2 min after the addition of the enzyme 0.2 mL of this media was diluted in 1.8 mL of a solution at 0 °C containing 60 mM Tris–maleate buffer, at different pH values, 1 mM ADP, and different $CaCl_2$ and EGTA concentrations to yield the free calcium concentration shown in the figure. The reaction was arrested 3 min after the dilution by the addition of $HClO_4$. The pH of the media after dilution was 8.0 (○), 7.0 (●), or 6.2 (△).

Ca^{2+}-ATPase are correlated with access of water into the catalytic site of the enzyme. The results reported here can be interpreted in similar terms. As expected, the phosphorylation of the enzyme by P_i was facilitated when the hydrophobicity of the medium was increased by addition of organic solvents and, once the phosphoenzyme was formed, synthesis of ATP was only observed if the concentration of organic solvent was decreased (Figures 10 and 11). However, the present study does not provide direct evidence that Me_2SO indeed impairs the entry of water into the catalytic site (Figures 10 and 11A). This will depend at least on the amount of water which does not interact with Me_2SO and on the difference of energy between the interaction of water with Me_2SO and of water with the chemical species available to the catalytic site.

Effect of Temperature and pH on the Ca^{2+} Requirement of ATP Synthesis. The difference between the low- and high-affinity binding sites determines the magnitude of the Ca^{2+} gradient required for the synthesis of ATP (de Meis & Tume, 1977; Verjovski-Almeida & de Meis, 1977; Trotta & de Meis, 1978). The data in Figures 12 and 13 show that at 0 °C the difference in affinity between the two sites decreased as the pH of the medium was raised, and at pH 8.0 a point was reached where the two affinities became practically equal. Under these conditions the enzyme appears to be able to drive the synthesis of ATP without the need of a significant Ca^{2+} transmembrane gradient. Two alternative explanations can account for this finding.

(a) At 0 °C and pH 8.0, the translocation of the Ca^{2+} binding site from the inner surface to the outer surface of the membrane is no longer accompanied by a significant change in its affinity for Ca^{2+}.

(b) The translocation of the Ca^{2+} binding site through the membrane is accompanied by a large variation in its affinity, but at 0 °C and pH 8.0 the binding site without Ca^{2+} bound would be translocated through the membrane. Thus *E–P would be spontaneously converted into E–P without the necessity of having a high Ca^{2+} concentration in the vesicle lumen. Once E–P is formed, the Ca^{2+} binding site would be already facing the outer surface of the membrane and have a high affinity for Ca^{2+}. Thus, the same Ca^{2+} concentration which activates the hydrolysis of ATP will also permit the transfer of phosphate from the phosphoenzyme to ADP.

$$^{*}E\text{-}P \rightleftarrows E\text{-}P$$

$$E\text{-}P + Ca_{out} \rightleftarrows E\text{-}P\cdot Ca_{out}$$

$$E\text{-}P\cdot Ca_{out} + ADP \rightleftarrows E\cdot Ca_{out} + ATP$$

It should be remembered that in the absence of organic solvent the enzyme is not phosphorylated by P_i at 0 °C and pH 8.0. The above discussion is concerned with the events required for the synthesis of ATP which occur after the enzyme is phosphorylated by P_i, i.e., with the Ca^{2+} requirement for the conversion of the phosphoenzyme from low into high energy.

The Ca^{2+} binding site and the catalytic site appear to be located in different parts of the enzyme molecule, and the interaction of ligands in one site promotes a conformational change in the enzyme which determines the properties of the other site (Shamoo & MacLennan, 1974; de Meis & Carvalho, 1976; Coan & Inesi, 1977; Shamoo & Goldstein, 1977; Champeil et al., 1978; Dupont, 1979a,b).

In this (Figure 12) and previous reports (Kanazawa, 1975; de Meis & Tume, 1977; Verjovski-Almeida & de Meis, 1977), it has been shown that above 20 °C the affinity of the two Ca^{2+} binding sites varies with the pH of the medium. The & Hasselbach (1977) reported that organic solvents do not modify the affinity of the enzyme for Ca^{2+}. In contrast, Figure 9A shows that in the presence of organic solvents Mg^{2+} can compete with Ca^{2+} for its binding site, but in the presence of a reduced Mg^{2+} concentration, organic solvents do not modify its affinity for Ca^{2+}. Therefore, it appears that within the Ca^{2+} binding site, electrostatic interactions are the main events which will determine the conformation state of the enzyme. This paper shows that the enzyme can be phosphorylated by P_i at any desired pH or temperature provided that the hydrophobicity of the medium is modified with the use of organic solvents. In the presence of an optimum Ca^{2+} concentration, the transfer of phosphate from the phosphoenzyme to ADP is impaired when the hydrophobicity of the medium is increased (Figure 10). The ATPase activity of the enzyme varies depending on the temperature of the medium and for each given temperature is inhibited by the addition of organic solvents (Table IV). At low temperature the conversion of the phosphoenzyme from low to high energy appears to be independent of the Ca^{2+} binding site of low affinity (Figure 12). It is well-known that the organization of water varies with the temperature. It therefore seems that within the catalytic site distribution and organization of water will vary depending on the conformational state of the enzyme.

Stabilization of the Phosphoenzyme by Mg^{2+}. In the absence of organic solvent, free P_i and not the complex P_i·Mg is the substrate of the Ca^{2+}-dependent ATPase (Punzengruber et al., 1978; Kolassa et al., 1979; Prager et al., 1979). The enzyme forms a ternary complex with P_i and Mg^{2+} (E·Pi·Mg) where the binding of one ionic species to the enzyme facilitates the binding of the other species. The ternary complex is in equilibrium with the phosphoprotein (E–P·Mg). The data of Figure 4 and Table III can be interpreted according to this model. A comparison of Tables II and III indicates that the effect of Me_2SO in increasing the apparent affinity for P_i is more pronounced than its effect in increasing the affinity of the enzyme for Mg^{2+}.

Early reports have shown that Mg^{2+} accelerates the rate of hydrolysis of the phosphoenzyme formed by ATP (Inesi et al., 1970; Kanazawa et al., 1971; Panet et al., 1971; Yamada & Tonomura, 1972; Garrahan et al., 1976). When these data were reported, it was not established whether the catalytic cycle of ATP hydrolysis included different forms of phosphoenzyme. In the reaction sequence (Figure 1) the interconversion of these forms is represented by reactions 4, 5, and 6. In the results reported in Figures 6 and 7, the only phosphoenzyme formed is $^{*}E$–P (reaction 6). The rate of hydrolysis of $^{*}E$–P was not accelerated by Mg^{2+} and, indeed, depending on the pH of the medium, hydrolysis could be reduced by Mg^{2+}. This suggests that the binding of Mg^{2+} to the enzyme may activate the rate of ATP hydrolysis, accelerating both the rate of conversion of $(Ca\cdot)_2$E–P into $^{*}E$–P (reactions 4 and 5) and, after the hydrolysis of $^{*}E$–P, the rate of conversion of $^{*}E$ into E (reaction 8). At present we do not know why the effect of Mg^{2+} on the rate of $^{*}E$–P hydrolysis varies with the pH of the medium (Figure 6).

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Reaction of Cytochrome *c* Oxidase with Endogenous and Exogenous Cytochrome *c*†

Lucile Smith,* Helen C. Davies, and María Elena Nava

ABSTRACT: The reaction of the membrane-bound cytochrome *c* oxidase of beef heart submitochondrial particles with added exogenous cytochrome *c*, which can diffuse off into the assay medium, was compared with the reaction with the endogenous cytochrome *c*, which is restrained on or within membrane vesicles. Our suspensions of particles appeared to contain about equal quantities of vesicles with the oxidase sites exposed (right-side out) and with the oxidase sites within the vesicles (inside out). Both showed similar variations in activity, measured polarographically with *N,N,N′,N′*-tetramethylphenylenediamine (TMPD) plus ascorbate, with an increase in pH of nonbinding buffers between pH 6 and pH 8. At pH 7.8 low concentrations of exogenous cytochrome *c* (0.05–0.1 μM) appeared to form a tight combination with the exposed oxidase sites, similar in reactivity to that with the endogenous cytochrome *c*. The data confirm our previous postulate [Smith, L., Davies, H. C., & Nava, M. E. (1979) *Biochemistry 18*, 3140] of the formation of an especially reactive combination of cytochrome *c* with cytochrome *c* oxidase under these conditions. In phosphate buffers the reaction with exogenous, but not with endogenous, cytochrome *c* was inhibited at all pH values above 6. We saw no evidence for accumulation of Würster's Blue within the vesicles under our experimental conditions when concentrations of TMPD below 0.75 mM were used in the polarographic assays. However, increased rates of O_2 uptake were observed with the reaction with endogenous cytochrome *c* but not that with exogenous cytochrome *c* at higher concentrations of TMPD. Concentrations of exogenous cytochrome *c* above 0.1 μM gave increased rates of O_2 uptake with the exposed vesicular oxidase similar to those seen previously with nonvesicular oxidase preparations, but higher concentrations of cytochrome *c* within the vesicles did not lead to a significant increase in O_2 uptake rates.

We have made detailed studies of the kinetics of reaction of soluble beef cytochrome *c* with several different kinds of preparations of beef cytochrome oxidase, using both spectrophotometric and polarographic methods (Smith et al., 1979a,b). These gave evidence for a combination of cytochrome *c* with cytochrome oxidase with a high turnover rate at low concentrations of cytochrome *c* (0.05–0.2 μM) when the assays were run polarographically with TMPD[1] plus ascorbate in Tris–cacodylate buffer, pH 7.8. Under other experimental conditions the polarographic assays and under all conditions the spectrophotometric assays gave lower turnover rates. In the spectrophotometric measurements of oxidase activity with soluble ferrocytochrome *c*, the ferricytochrome *c* formed must dissociate away into solution in order for another molecule to react.

To obtain more insight into the cytochrome *c*–cytochrome oxidase combination, we have compared the reaction of the oxidase with soluble exogenous cytochrome *c*, which can diffuse off into the suspending medium, with the endogenous cytochrome *c* of mitochondrial membrane vesicles, which cannot. The preparation of submitochondrial particles, made by sonication of heart mitochondria, contains a mixture of vesicles, some with the cytochrome *c* reaction site exposed and others with the sites within the interior.

Polarographic assays described here with 0.75 mM TMPD plus ascorbate of the reactions with endogenous and with added exogenous 0.1 μM cytochrome *c* show similar activities and also similar changes with variation of pH of Tris–cacodylate or Hepes buffers. Both reactions have low activity at pH 6 and maximal activity at pH 7.8. Ions which bind to cytochrome *c*, such as phosphate (Stellwagen & Shulman, 1973), inhibit the reaction with exogenous but not with endogenous

† From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755, and the Department of Microbiology, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104. *Received February 12, 1980.* This research was supported by Grant GM-06270 from the National Institutes of Health and by General Research Support Grant RR-05392 to L.S. and by Biomedical Research Support Grant RR-07083-14 to H.C.D.

[1] Abbreviations used: SMP, submitochondrial particles; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

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