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Dimethyl sulfoxide: a possible effect on the interconversion of phosphorylated forms of Na^+,K^+-ATPase

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Purified Na^+,K^+-ATPase from kidney outer medulla was phosphorylated by P_i in a reaction synergistically stimulated by Mg^{2+}, when 40% (v/v) dimethyl sulfoxide was added to the assay medium. The phosphoenzyme formed at this solvent concentration was able to synthesize ATP even in the presence of Mg^{2+}, because hydrolysis was impaired. ATP $\rightleftharpoons$ [^{32}P]P_i exchange was also inhibited, indicating that partial reactions in the forward direction were blocked by the solvent. In 40% (v/v) dimethyl sulfoxide the enzyme's affinity for ADP decreased, in comparison with the values observed in purely aqueous medium. Addition of K^+, which accelerated dephosphorylation of Na^+,K^+-ATPase in a totally water medium, partially reversed the inhibition of hydrolysis that was observed in the presence of dimethyl sulfoxide.

Introduction

The study of partial reactions catalyzed by the Na^+,K^+-ATPase in a purely aqueous medium indicates that during its catalytic cycle, the enzyme goes through conformational changes that include two phosphorylated forms, E_1-P and E_2-P [1–6]. It has been established that in the presence of Na^+ and with ATP as substrate, E_1-P is formed. As the reaction proceeds to the steady state, E_1-P is converted into E_2-P, which is hydrolyzed. In the reverse direction, the enzyme is phosphorylated by P_i in the presence of Mg^{2+} [1,2,7]. When this reaction is measured in a medium containing the organic solvent dimethyl sulfoxide (Me_2SO) and low P_i concentration, the amount of phosphoenzyme obtained at equilibrium is higher than that obtained in the absence of the solvent [7]. The E_2-P formed can be interconverted to E_1-P in the absence or in the presence of the solvent, if a high concentration of Na^+ is supplied to the reaction medium. It has been demonstrated that the Na^+,K^+-ATPase previously phosphorylated by P_i transfers its phosphate to ADP, synthesizing ATP either in a purely aqueous medium [1,2] or in the presence of 40% (v/v) Me_2SO [7], after sequential addition of Na^+ and ADP to the medium.

A low Mg^{2+} concentration is required for the enzyme to be phosphorylated by ATP. This ion at high concentration seems to favor the interconversion from E_1-P to E_2-P, inhibiting ATP $\rightleftharpoons$ ADP exchange and activating hydrolysis [1,3,5]. This effect of Mg^{2+} may explain why there is no ATP synthesis in a totally aqueous medium, unless the ion is chelated by CDTA [2]. In contrast, in 40% (v/v) Me_2SO the Na^+,K^+-ATPase can catalyze ATP formation, even with Mg^{2+} in the medium [7].

In order to investigate the effect of dimethyl sulfoxide on the interconversion of phosphorylated intermediates, partial reactions of Na^+,K^+-ATPase were analyzed both in the absence and in the presence of the solvent. The results indicated that addition of 30–50% (v/v) Me_2SO favored the E_2-P to E_1-P conversion, in opposition to the Mg^{2+} effect. These data suggest that changes in water activity of the medium interfere with the interconversion of the phosphorylated forms of the Na^+,K^+-ATPase. This fact may be correlated with a hydrophobic/hydrophilic transition during the catalytic cycle of the enzyme, as previously proposed [7,8].

Methods

The Na^+,K^+-ATPase was prepared from sheep kidney using the method of Jørgensen [9] as modified by Munson [10], the specific activity varied between 15 and

Abbreviations: Tris, tris(hydroxymethyl)aminomethane: CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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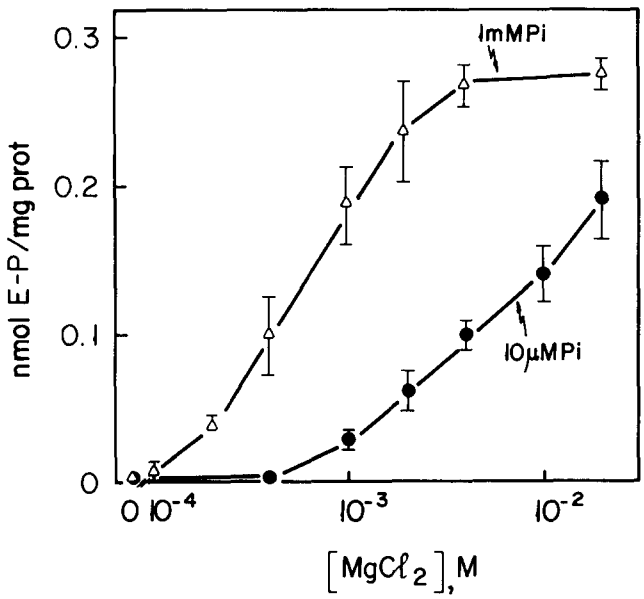


Fig. 1. Mg^{2+} dependence of E-P formation. The assay medium contained 50 mM Tris-maleate (pH 7), 40% (v/v) Me_2SO and (●) 10 μM P_i or (△) 1 mM P_i. The final volume was 1 ml. The reaction was started by addition of the enzyme to a final concentration of 1.0 mg protein/ml and stopped after 2 min at 37 °C with 1 vol. of a cold 10% trichloroacetic acid solution. The values shown are the averages ± S.E. of five experiments.

20 μmol/min per mg protein. The [^{32}P]P_i was purified as described elsewhere [11]. Phosphorylation by P_i, ATP synthesis and identification of ATP formed [7], ATPase activity and ATP ⇌ P_i exchange reactions [12] were measured as previously described. The maximal level of phosphoenzyme formed from P_i varied among the different enzyme preparations used. Therefore, each set of experiments was performed with the same enzyme preparation. In Fig. 4B, ADP was pre-incubated for 1 h at room temperature with 20 U hexokinase and 40 mM glucose before being added to the reaction medium. Other conditions used are indicated in the figure legends. Me_2SO, ATP, ADP and hexokinase (EC 2.7.1.1) were from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade.

Results

Magnesium is a requirement for phosphorylation of the Na^+,K^+-ATPase by P_i in Me_2SO-containing medium (Fig. 1), as previously observed in totally aqueous medium [1,2]. In 40% (v/v) Me_2SO, the concentration of Mg^{2+} needed to reach any given level of enzyme phosphorylation varied with the P_i concentration used, being lower the higher the P_i concentration. These results indicate that the binding of Mg^{2+} to the enzyme influences the reaction with P_i, in the phosphoenzyme formation. The same effect was also observed in purely aqueous medium (Table I). But, in this case, to obtain the same level of phosphoenzyme with 1 mM P_i, 40 mM

TABLE I

Effect of Mg^{2+} on phosphorylation of the Na^+,K^+-ATPase by P_i without or with Me_2SO

The procedure was the same as that described in Fig. 1, the values are the averages of five experiments, all with a S.E. ≤10% of the mean.

[Mg^{2+}] mM	Phosphorylation by P_i (nmol E-P·mg^{-1})			
	in purely aqueous medium with		in 40% Me_2SO with	
	200 μM P_i	1 mM P_i	20 μM P_i	1 mM P_i
0.4	0.09	0.20	0.01	0.18
2.0	0.34	0.46	0.07	0.64
20	0.60	0.54	0.43	1.13
40	0.42	0.63	0.54	1.17

Mg^{2+} was needed in completely aqueous medium and only 2 mM Mg^{2+} with Me^2SO. The phosphorylation of the Ca^{2+}-ATPase from sarcoplasmic reticulum by P_i shows a similar Mg^{2+} requirement both in a purely aqueous medium [13] and in the presence of organic solvent [14]. The kinetic data obtained by Punzengruber et al. [13] indicate that for Ca^{2+}-ATPase the binding of the first component, P_i or Mg^{2+}, to the enzyme influences the binding of the second component to form the ternary complex that leads to the phosphoenzyme formation.

Phosphorylation of Na^+,K^+-ATPase by P_i, in the presence of a fixed Mg^{2+} concentration, shows that for a given P_i concentration more phosphoenzyme was formed when the organic solvent was present in the assay medium (Fig. 2). Comparing the phosphorylation in this case with that measured in a purely aqueous medium, it can be seen that in 40% (v/v) Me_2SO the same level of phosphoenzyme was attained with 0.25 mM as with 2 mM P_i and the level did not change with pH. On the other hand, in a purely water medium the

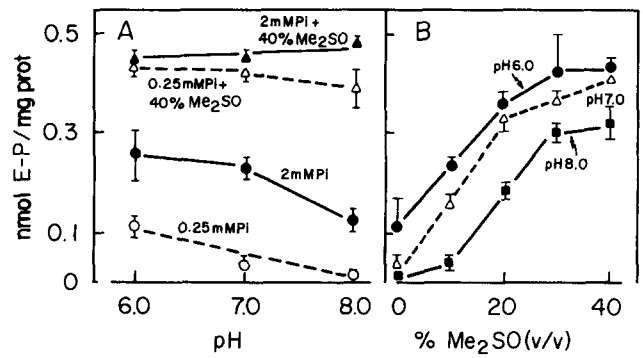


Fig. 2. Me_2SO effects on E-P formation at different pH values. The assay medium contained 10 mM $MgCl_2$. (A) 50 mM Tris-maleate at pH values shown on abscissa, 0.25 mM [^{32}P]P_i (△, ○) or 2 mM [^{32}P]P_i (▲, ●) in purely aqueous medium (○, ●) or in 40% (v/v) Me_2SO (△, ▲). (B) 50 mM Tris-maleate at pH 6 (●), pH 7 (△), pH 8 (■) and 0.25 mM [^{32}P]P_i. The reaction was performed as described in Fig. 1. The values shown are the averages ± S.E. of four experiments.

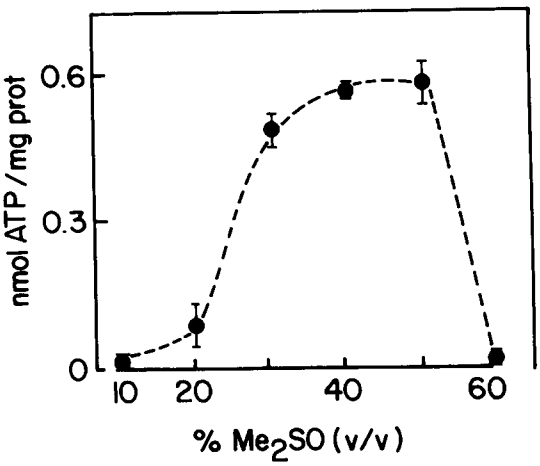


Fig. 3. ATP synthesis in the presence of Me_2SO. The enzyme (1.5 mg/ml) was phosphorylated at 37 °C in a medium containing 50 mM Tris-maleate (pH 7), 2 mM $MgCl_2$, 0.2 mM [^{32}P]P_i and 40% (v/v) Me_2SO. The final volume was 0.5 ml. After 2 min, this mixture was diluted in 1.5 ml of a solution containing 50 mM Tris-maleate (pH 7), 2 mM $MgCl_2$, 0.2 mM [^{32}P]P_i, 400 mM NaCl, 1 mM ADP and the Me_2SO concentrations shown on the abscissa. After 1 min at 37 °C the reaction was stopped with 0.1 ml of cold trichloroacetic acid 100% (w/v).

lower of these two concentrations was insufficient to phosphorylate the enzyme at pH 7 and 8. This effect of the co-solvent can be explained by the increase in the enzyme affinity for P_i that occurs in the presence of Me_2SO [7]. The radioactive phosphoenzyme formed in 40% (v/v) Me_2SO is in dynamic equilibrium with the P_i of the medium. Addition of cold P_i or Na^+ produced a phosphoenzyme decay (data not shown). In Me_2SO-containing medium, addition of Na^+ probably displaced the equilibrium towards E_1, a conformation that does not accept P_i, as in purely water medium [2,5].

In a previous report [7], it was observed that the Na^+,K^+-ATPase phosphorylated with P_i and Mg^{2+} in an assay medium with 40% (v/v) Me_2SO, transferred its phosphate to ADP, synthesizing ATP. This was achieved after addition of a mixture containing Na^+, ADP and the same solvent concentration used in the phosphorylation reaction. In order to understand this requirement for Me_2SO in ATP formation, synthesis was performed in the presence of different solvent concentrations (Fig. 3). This figure shows that net synthesis of ATP varied according to the Me_2SO/water ratio used, being lower the lower the co-solvent concentration. As ATP formation decreased with the increase in water content in the assay mixture, synthesis was then performed in the absence of the solvent. In this case, the enzyme was phosphorylated by P_i in a purely aqueous medium. Fig. 4A shows that the phosphoenzyme hydrolysis was accompanied by the appearance of radioactive ATP, identified as described elsewhere [7]. But, the amount of ATP formed did not correspond to the phosphoenzyme decay. When the synthesis reaction was coupled to a hexokinase-glucose system, the phosphoenzyme disappeared at the same rate, but no ATP could be detected as glucose 6-phosphate (Fig. 4B). One interpretation of these results would be the occurrence in Fig. 4A of an ATP ⇌ [^{32}P]P_i exchange reaction, generating [γ-^{32}P]ATP transiently. The cold ATP required for hydrolysis during the exchange reaction must come from the commercial ADP [1], since the exchange is eliminated (Fig. 4B) by preincubation of ADP with the hexokinase-glucose system (see Methods). In a previous report [12], it was shown that the ATP ⇌ [^{32}P]P_i exchange occurred in an assay medium similar to that used to measure net synthesis of ATP in a totally aqueous medium. In 40% (v/v) Me_2SO, on the other hand, the decay of the phosphoenzyme was closely coupled to the appearance of ATP [7]. The same result was obtained in the absence of the hexokinase-glucose system (data not shown). The results obtained in the experimental conditions of Figs. 4A and 4B are different from those reported by Post et al. [2], who were able to measure net synthesis of ATP in a purely aqueous medium in synchrony with the phosphoenzyme decay. One difference between our assay conditions and those of Post et al. is the presence of Mg^{2+} in our experiments even in the synthesis step. To measure ATP synthesis, Post et al. worked at 0 °C and added CDTA after the enzyme be phosphorylated by P_i. In fact, Table II shows that in purely aqueous medium at 0 °C, ATP synthesis could only be measured after removal of the Mg^{2+} from the assay medium with CDTA. Conversely, the level of ATP synthesized in 40% (v/v) Me_2SO was

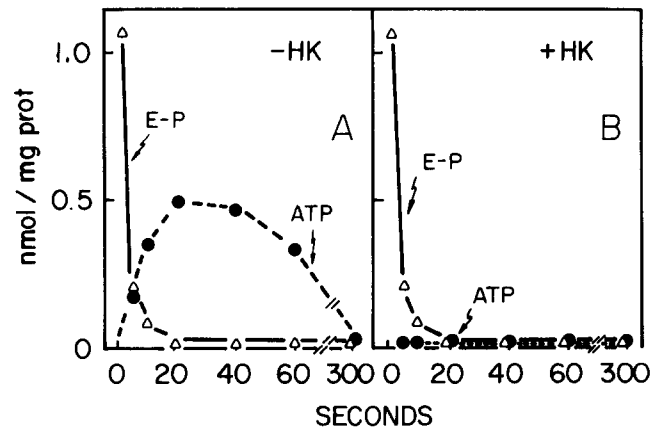


Fig. 4. ATP synthesis in a purely aqueous medium. The enzyme (1.5 mg/ml) was phosphorylated at 37 °C in a medium containing 50 mM Tris-maleate (pH 7), 20 mM $MgCl_2$, 2 mM [^{32}P]P_i. The final volume was 0.5 ml. After 2 min, this mixture was diluted in 1.5 ml of a solution containing 50 mM Tris-maleate (pH 7), 400 mM NaCl, 1 mM ADP, 20 mM $MgCl_2$ and 2 mM [^{32}P]P_i. In (B), ATP synthesis was performed in the presence of 20 U of hexokinase and 40 mM glucose and the ADP was pre-incubated with these two components (see Methods) to eliminate ATP contaminating. At each time interval the reaction was stopped as described in Fig. 3. △, phosphoenzyme; ●, ATP synthesized.

TABLE II

ATP synthesis in the absence or in the presence of Me_2SO and CDTA

The procedure was the same as that described in Fig. 3. Phosphorylation was performed with 1 mM P_i, 1.5 mM $MgCl_2$ and Tris-maleate (pH 7) without or with 40% (v/v) Me_2SO. After 4 s at 0 °C or 2 min at 37 °C the mixture was diluted as described in Fig. 3 in absence or in the presence of 40% (v/v) Me_2SO. The values are the average of four determinations, with S.E. within 10% of the mean value.

Phosphorylation by P_i		ATP synthesis		
Assay [a] conditions	nmol E-P·mg^{-1}	Assay [b] conditions	nmol ATP·mg^{-1}	
			−CDTA	+CDTA (20 mM)
H_2O, 0 °C, 4 s	0.22	H_2O, 0 °C, 6s	0.03	0.20
Me_2SO, 0 °C, 4 s	0.40	Me_2SO, 0 °C, 6 s	0.40	0.40
Me_2SO, 37 °C, 2 min	0.96	Me_2SO, 37 °C, 1 min	1.00	1.00

[a] Phosphorylation was performed with Tris-maleate (pH 7), 1 mM P_i and 1.5 mM $MgCl_2$, without or with 40 % (v/v) Me_2SO.

[b] ATP synthesis conditions were 400 mM NaCl, 1 mM ADP and Tris-maleate (pH 7), without or with 40% (v/v) Me_2SO.

the same either in the absence or in the presence of CDTA, both at 0 °C or at 37 °C. At this highest temperature in a purely aqueous medium ATP formation occurred from which it can be inferred that there was ATP ⇌ P_i exchange (Fig. 4A); the same was not seen at 0 °C probably due to the decrease of the enzyme turnover (Table II).

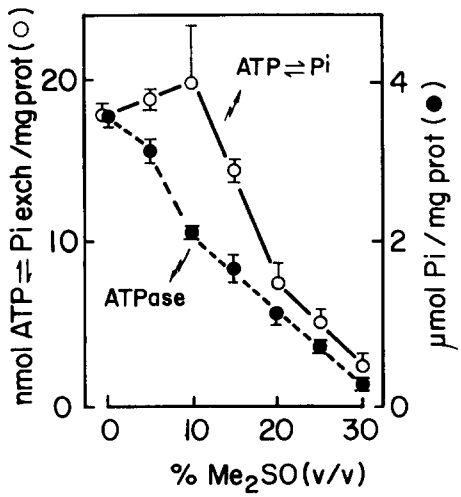


Fig. 5. Effect of Me_2SO on Na^+-ATPase activity and ATP ⇌ P_i exchange. The assay medium composition was 50 mM Tris-maleate (pH 7.4), 1 mM ATP, 0.2 mM ADP, 10 mM $MgCl_2$, 5 mM P_i, 400 mM NaCl, 25 µg of purified protein and Me_2SO as shown on the abscissa. The final volume was 0.2 ml. For the ATPase activity (●), [γ-^{32}P]ATP and non-radioactive P_i were used. For ATP ⇌ P_i exchange (○), [^{32}P]P_i and non-radioactive ATP were used. The reaction was started by the addition of the enzyme and stopped with 0.05 ml of 50% cold trichloroacetic acid solution after 15 min at 37 °C. The values shown are the averages ± S.E. of three experiments.

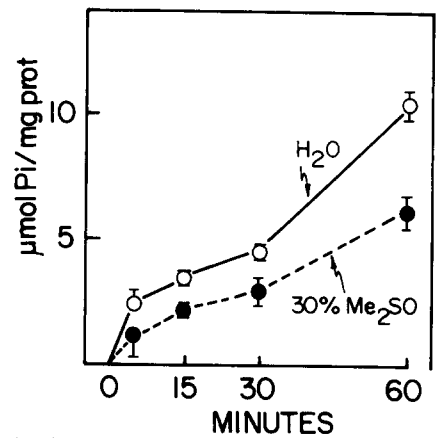


Fig. 6. Na^+,K^+-ATPase activity in the absence or in the presence of Me_2SO. The enzymatic activity was assayed in the same conditions used in Fig. 5 for Na^+-ATPase activity, except that here 100 mM KCl was added to the medium, in the absence (○) or in the presence of 30% (v/v) Me_2SO (●). The values are the averages ± S.E. of three experiments.

The effect of the co-solvent on ATP synthesis may be attributed to an inhibition of ATP hydrolysis. Thus, these reactions were analyzed simultaneously by measuring the Na^+-ATPase activity and ATP ⇌ P_i exchange, in the presence of increasing Me_2SO concentrations. The assay conditions included a high Na^+ concentration and no K^+ [12]. Fig. 5 shows that both hydrolysis and exchange were progressively inhibited by increasing Me_2SO concentrations, in contrast to what

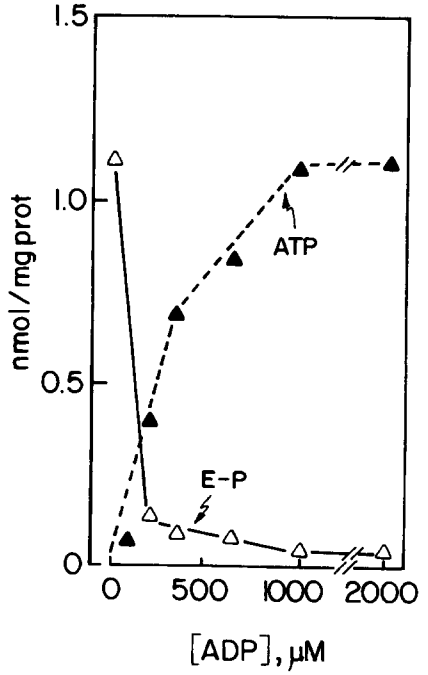


Fig. 7. ADP dependence of ATP synthesis in the presence of Me_2SO. The enzyme was phosphorylated as described in Fig. 3. Synthesis was performed in 40% (v/v) Me_2SO, 0.2 mM P_i, 2 mM MgCl, 400 mM NaCl, 50 mM Tris-maleate (pH 7) and different concentrations of ADP. △, phosphoenzyme; ▲, ATP synthesized.

was observed with net synthesis of ATP (Fig. 3). In the presence of both ions Na^+ and K^+ hydrolysis was still inhibited (Fig. 6). In this case, however, Me_2SO was able to inhibit ATPase activity by only 40% or less.

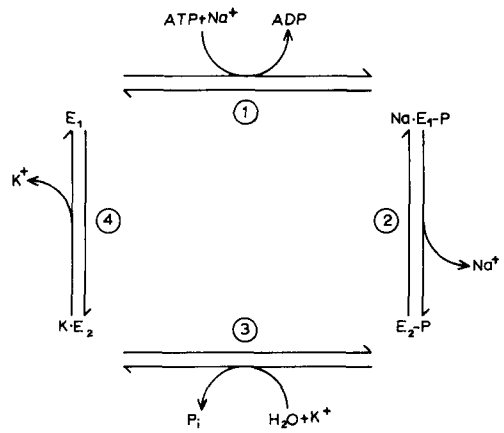
In 40% (v/v) Me_2SO the phosphoenzyme required more ADP to form ATP. Whereas in totally aqueous medium the apparent K_m for ADP was 12 μm [1], in 40% (v/v) Me_2SO it increased to 300 μM ADP (Fig. 7).

Discussion

In a totally aqueous medium, partial reactions catalyzed by the Na^+,K^+-ATPase can be discussed on the basis of the sequence shown in Scheme I, proposed by Post et al. [2]:

According to this scheme, ATP can be formed by reversal of steps 3, 2 and 1. In fact, in Me_2SO or in a totally aqueous medium, the addition of Na^+ and ADP to a medium containing the phosphorylated enzyme resulted in ATP synthesis (Figs. 3 and 7; Refs. 1, 2 and 7).

Addition of Me_2SO favors ATP synthesis and inhibits ATP hydrolysis. Furthermore, with 30% to 50% (v/v) Me_2SO, ATP can be formed also in the presence of Mg^{2+} (Fig. 3, Table II) which is not the case in purely water medium (Table II). The effect of this ion on the intermediary steps of the catalytic cycle of the Na^+,K^+-ATPase has raised the discussion as to whether Mg^{2+} stimulates or not the E_1-P to E_2-P transition [5,15,16]. The data of Fukushima and Post [17] showed that this ion becomes firmly bound to the enzyme when it is phosphorylated by ATP and is released only later when E_2-P is hydrolyzed. This may be the reason why ATP was not formed in pure water in our assay conditions, unless CDTA was added to the medium (Fig. 4, Table II). The ATP synthesis observed with Me_2SO and Mg^{2+} present, can be explained if the solvent stimulates the E_2-P to E_1-P transition in opposition to the effect of the Mg^{2+} in the forward direction. The inhibition of the Na^+-ATPase activity observed in the presence of Me_2SO



Scheme I.

(Fig. 5) and the 2–3-fold increase in the enzyme affinity for Na^+ in ATP synthesis [7], support this interpretation. In the first case, the effect of Me_2SO on step 2 would impair the E_1-P to E_2-P transition, thus accounting for the inhibition of hydrolysis seen in Figure 5. Moreover, less Na^+ would be required to transform E_2-P to E_1-P because step 2 is driven in the same direction by the solvent. The decrease in the enzyme turnover may explain why ATP $\rightleftharpoons$ [^{32}P]P_i exchange is progressively inhibited when Me_2SO concentration is increased from 10% to 30% (Fig. 5). The levels of [γ-^{32}P]ATP recovered in continuous cycles of synthesis measured during 15 min at 30% Me_2SO (2.6 nmol/mg) as observed in Fig. 5, were 2–3-times higher than those measured in a single cycle of synthesis (Fig. 3, Table II). These results show that the enzyme should perform fewer cycles of synthesis as the solvent concentration is increasing in the medium. Moreover, ATP $\rightleftharpoons$ [^{32}P]P_i exchange could be additionally impaired due to a decrease in the enzyme affinity for ADP promoted by Me_2SO (Fig. 7, compare with Fig. 6 in Ref. 1).

In the presence of K^+, however, the stimulation of the interconversion of E_2-P to E_1-P promoted by Me_2SO would be greatly reduced because E_2-P would rapidly be dephosphorylated to form E_2, which in turn would be converted to E_1 in step 4, a reaction that is stimulated by the ATP present in the medium. These effects of K^+ and ATP on the catalytic cycle of the Na^+,K^+-ATPase have been described for totally aqueous medium [18]. Indeed, when ATP hydrolysis was measured with Na^+ and K^+ present, Me_2SO was far less inhibitory than when only Na^+ was present (compare Figs. 5 and 6). The fact that K^+ only partially reverses the solvent effect on hydrolysis can be attributed to the increase in the enzyme affinity for P_i observed in Me_2SO, with stabilization of the E_2-P form (Fig. 2).

The results also show that the effects of Me_2SO on partial reactions of the Na^+,K^+-ATPase are qualitatively different according to the solvent concentration used. This may indicate that variation in water activity can favor different steps of the catalytic cycle of the enzyme. According to Dupont and Pougeois [19], water activity varies from 0.9 to 0.54 as Me_2SO is increased from 10% to 40% (v/v) Me_2SO. For example, with 10% (v/v) Me_2SO ATP $\rightleftharpoons$ [^{32}P]P_i exchange was slightly stimulated whereas Na^+-ATPase activity decreased to 50% of the value without solvent (Fig. 5). With respect to phosphorylation by P_i, the results indicate that at 10% (v/v) Me_2SO the E_2 form of the enzyme predominates and thus the reversal of step 3 is favored (Fig. 2). In this case ATP is not formed because the Mg^{2+} concentration in the medium does not permit the transition from E_2-P to E_1-P form. As the concentration of Me_2SO is increased to 30%–50%, the effect of Mg^{2+} is overcome and the step 2 is stimulated in the backward direction (Fig. 3, Table II). The same effect of

increasing Me_2SO concentration is observed with the K^+-nitrophenylphosphatase activity of the Na^+,K^+-ATPase, which is related to the E_2 form of the enzyme [20]. In a study of the kinetic parameters of this reaction, it was found that it was stimulated by 10% (v/v) Me_2SO and blocked when the concentration was raised to 30% to 50% (v/v) Me_2SO [21]. Although facilitating the E_2-P to E_1-P conversion, at these solvent concentrations the E_1-P form of Na^+,K^+-ATPase would be stabilized, impairing the transfer of the phosphate from the enzyme to ADP. This may be the reason why the Na^+,K^+-ATPase needs 25-fold more ADP to synthesize ATP in 40% (v/v) Me_2SO (Fig. 7), than in a purely aqueous medium [1]. This would also explain why there is no synthesis above 50% (v/v) Me_2SO (Fig. 3).

The effects of Me_2SO analyzed here are consistent with the proposal that the Na^+,K^+-ATPase in the E_2 form has a catalytic site with hydrophobic characteristics, as discussed before [7.8]. They are also indicating that a hydrophobic/hydrophilic transition occurs during the catalytic cycle of the enzyme.

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