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STUDY ON THE DIFFERENTIAL MODIFICATIONS OF $(Na^+ + K^+)$-ATPase AND ITS PARTIAL REACTIONS BY DIMETHYLSULFOXIDE

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SUMMARY

1. Dimethylsulfoxide inhibits $(Na^+ + K^+)$-activated ATPase but increases [^{14}C]ADP binding to the enzyme and stimulates the associated K^+-dependent *p*-nitrophenylphosphatase.

2. Enhancement of [^{14}C]ADP binding occurs by a decrease of the dissociation constant from $K_D = 0.95\ \mu M$ to $0.09\ \mu M$ in the presence of 30% dimethylsulfoxide. 5 mM Na^+ enhances ADP affinity in the presence and absence of dimethylsulfoxide about 3 fold. However, the decrease of the ADP affinity in the presence of K^+ is abolished by increasing concentrations of dimethylsulfoxide. Two ADP-binding sites, a high-affinity ($K_D = 0.17\ \mu M$) and a low-affinity site ($K_D = 0.40\ \mu M$), appeared in the presence of 30% dimethylsulfoxide and 0.2 mM Mg^{2+} or Ca^{2+}. Mg^{2+} and Ca^{2+} decreased the affinity of the enzyme for ADP in the presence and absence of dimethylsulfoxide.

3. Dimethylsulfoxide decreased the rate of Na^+-dependent phosphorylation of an EDTA-washed enzyme (E_1 conformation) and reduced the hydrolysis of the [^{32}P]phosphoprotein. However, dimethylsulfoxide enhanced the Na^+-dependent phosphorylation of the Rb^+-bearing dephosphoenzyme ($Rb^+ \cdot E_2$).

4. Dimethylsulfoxide increased the dissociation constant of the ouabain–receptor complex when ouabain binding was studied via the Na^+-dependent pathway.

5. The results suggest that dimethylsulfoxide acts on several sites: It favors the E_1 conformational state of the enzyme by an increased affinity for nucleotide triphosphates and diphosphates, by reducing the affinity of K^+ for the enzyme in the presence of nucleotides and possibly by an expulsion of K^+ from the K^+-dephosphoenzyme. Inhibition of $(Na^+ + K^+)$-ATPase may occur by a decreased interaction of the phosphokinase and phosphatase sites with the phosphorylacceptor site.

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INTRODUCTION

$(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, (E.C. 3.6.1.3)) catalyzing the active transport of Na^+ and K^+ through cell membranes [1, 2], is assumed to undergo conformational changes of the enzyme structure during the transport process. Dimethylsulfoxide which acts as a diuretic agent in animals [3], inhibits $(Na^+ + K^+)$-activated ATPase of cell membranes but activates a K^+-dependent *p*-nitrophenylphosphatase [4–6]. The latter enzyme is assumed to reflect the terminal K^+-dependent hydrolytic process of ATPase [7–9]. It has been suggested that the effects of dimethylsulfoxide on $(Na^+ + K^+)$-ATPase and K^+ phosphatase select or modify intermediary reactions or conformations [6].

If this were so, dimethylsulfoxide would be rather helpful in clarifying the events during $(Na^+ + K^+)$-activated ATP hydrolysis. In order to obtain additional information on the mode of action of dimethylsulfoxide, we studied the influence of this substance on nucleotide binding, rates of phosphorylation and dephosphorylation and on the ouabain receptor interaction.

MATERIALS

[^{14}C]ADP was from Schwarz-Mann, Orangeburg, N.Y., USA (lithium salt spec. act. 288–359 Ci/mole) or from Amersham-Buchler, Braunschweig, Germany (ammonium salt, spec. act. 535 Ci/mole). [^{3}H]ouabain with a specific activity of 13 Ci/mmole was from New England Nuclear, Dreieichenhain, Germany. Triton X-100 was from Koch-Light Laboratories Ltd. Colnbrook, Bucks, England. All other reagents were of analytical grade and obtained from Boehringer, Mannheim or E. Merck A.G., Darmstadt, Germany.

METHODS

Preparation and quantitation of enzyme

$(Na^+ + K^+)$-activated ATPase from beef brain was prepared and quantitated as described previously [10]. One enzyme unit (U) is defined as the amount of enzyme hydrolyzing 1 μmole ATP per min at 37 °C. Protein was quantitated by the procedure of Lowry et al. [11].

Measurement of nucleotide binding capacity and ADP affinity

$(Na^+ + K^+)$-ATPase was converted into the E_1 form [12] by washing it twice with 10 mM Tris–EDTA and subsequently with distilled water. Nucleotide binding was measured with [^{14}C]ADP by a rapid mixing centrifugation method [13]. ADP is a competitive inhibitor of $(Na^+ + K^+)$-activated ATPase [14] and a co-substrate of the Na^+-dependent ATP–ADP transphosphorylation reaction, which is a partial reaction of the overall reaction [15]. The use of this test system has the advantage over the test using γ[^{32}P]ATP [16, 21] in that with [^{14}C]ADP the effects of Mg^{2+} on the nucleotide binding and affinity can also be studied. This is not possible with γ[^{32}P]ATP, because trace amounts of Mg^{2+} can hydrolyze ATP. [^{14}C]ADP binding to $(Na^+ + K^+)$-ATPase was studied as follows. After the incubation of 0.8–2.4 mg enzyme protein for 0.5–3 min at 0 °C with 50 mM Tris pH 7.4, 93–134 nM

[^{14}C]ADP and the additions mentioned in the figures in a total volume of 1 ml, the membranes were spun down at 70 000 $\times g$ at 4 °C in a rotor 50 Ti of a Spinco L50 ultracentrifuge. After careful removal of any remaining incubation medium from the walls of the polypropylene centrifuge tubes, the sediment was dissolved in 1 ml 1 M NaOH at 50 °C. The solubilized protein was neutralized with conc. HCl, dissolved in a scintillation solution containing Triton X-100 [16] and counted in a liquid scintillation spectrophotometer. Each experimental point was performed in duplicate or triplicate. All results were corrected for unspecific binding by subtracting the value obtained in the presence of 10 mM unlabeled ADP. Dissociation constants of the ADP–enzyme complex were calculated from the slopes of the lines of a Scatchard plot [34] (Figs 4, 5, 6, 8). The nucleotide binding capacity was obtained by extrapolation of the line to the ordinate.

Phosphorylation of enzyme protein from γ[^{32}P]ATP

2 mg EDTA-washed ($Na^+ + K^+$)-ATPase was incubated at 0 °C with 50 mM Tris-HCl pH 7.4, 50 μM γ[^{32}P]ATP, 50 μM MgCl and 100 mM NaCl in a total volume of 1 ml. Phosphorylation of enzyme protein was started with γ[^{32}P]ATP under rapid mixing. At the times indicated the reaction was terminated with 5 ml cold 5% trichloroacetic acid containing unlabeled ATP and P_i. After precipitation of the denatured protein with a bench centrifuge, the sediments were washed twice with 5 ml 5% cold trichloroacetic acid. The sediment was solubilized with 1 ml 1 M NaOH and treated as described for the nucleotide binding assay. All assays were corrected for a blank, where 100 mM NaCl was substituted by 10 mM KCl. Comparable results (Fig. 10A) were obtained when the Mg^{2+} concentration was raised to 1 mM.

The rate of dephosphorylation was measured as described by Post et al. [17]. After phosphorylation, the enzyme protein (2 mg) at 0 °C in 50 mM Tris–HCl pH 7.4, 40 μM MgCl, 40 μM γ[^{32}P]ATP and 20 mM NaCl in the presence or absence of 20% (v/v) dimethylsulfoxide for 30 s, phosphorylation was blocked by the addition of 2 μmoles unlabeled ATP. The spontaneous dephosphorylation was followed as a function of time by stopping the reaction by trichloroacetic acid at the times indicated. The phosphate incorporation after 30 s was set at 100% and the relative decrease of the [^{32}P]intermediate was calculated.

Rephosphorylation studies with γ[^{32}P]ATP were performed as described by Post et al. [18].

K^+-activated *p*-nitrophenylphosphatase was assayed by incubating 0.03–0.06 mg enzyme protein for 10 min at 37 °C in 50 mM Tris–HCl pH 7.8, 3 mM $MgCl_2$, 10 mM KCl, and 5 mM Tris–*p*-nitrophenylphosphate in a total volume of 1 ml. The reaction was stopped by the addition of 1 ml 1 M NaOH. The activity in the presence of 10^{-4} M ouabain was subtracted. Activity was calculated from the optical density at 405 nm.

The assay of ouabain binding was measured with the rapid centrifugation method described previously in detail [19].

RESULTS

Effect of dimethylsulfoxide and other solvents on $(Na^+ + K^+)$-ATPase and its partial reactions

In confirmation of the previous work [4–6], dimethylsulfoxide increased the activity of K^+-activated *p*-nitrophenylphosphatase, whilst $(Na^+ + K^+)$-activated ATPase was inhibited. In addition it was found that nucleotide binding is also increased in the presence of dimethylsulfoxide (Fig. 1A). Stimulation of K^+-activated *p*-nitrophenylphosphatase was also found with glycerol and diethyleneglycol. Since dimethylsulfoxide is an aprotic solvent, the effect of other aprotic solvents on $(Na^+ + K^+)$-ATPase and K^+-activated *p*-nitrophenylphosphatase were investigated. *N,N,N,N,N,N*-hexamethyl phosphoric triamide and dimethylformamide (Fig. 1B) inhibited both, $(Na^+ + K^+)$-ATPase and K^+-phosphatase activity [35]. However, [^{14}C]ADP binding to the nucleotide receptor was increased. A similar phenomenon was observed with ethyleneglycol and ethanol. Ethanol inhibits $(Na^+ + K^+)$-activated ATPase [20] and K^+-activated *p*-nitrophenylphosphatase (not shown) in a way competitive with K^+ [20]. So it appears that the effects of dimethylsulfoxide are not caused by the aprotic nature of this solvent. Furthermore, it is evident that dimethylsulfoxide acts in a way different to dimethylformamide [35]. The data also show that the nucleotide binding site of $(Na^+ + K^+)$-ATPase is relatively stable against organic solvents.

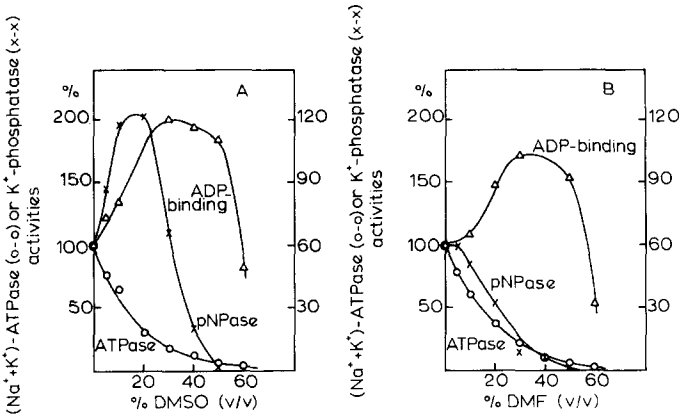


Fig. 1. Comparison of the action of dimethylsulfoxide and dimethylformamide on $(Na^+ + K^+)$-ATPase and its partial reactions. A. Effect of increasing dimethylsulfoxide (DMSO) concentrations. B. Effect of increasing dimethylformamide (DMF) concentrations. △—△, pmoles [^{14}C] ADP bound/mg protein.

Effects of dimethylsulfoxide on the nucleotide receptor

The nucleotide binding site of $(Na^+ + K^+)$-ATPase changes its affinity in the presence of univalent and divalent cations [13, 16, 21]. It was of interest therefore to see whether dimethylsulfoxide influences the cation sensitivity and binding capacity of the nucleotide binding site.

The increase in ADP binding with increasing concentrations of dimethylsulfoxide (Fig. 1) is caused by a decrease of the dissociation constant of the enzyme–ADP complex (Table I). The dissociation constant of the enzyme–ADP complex

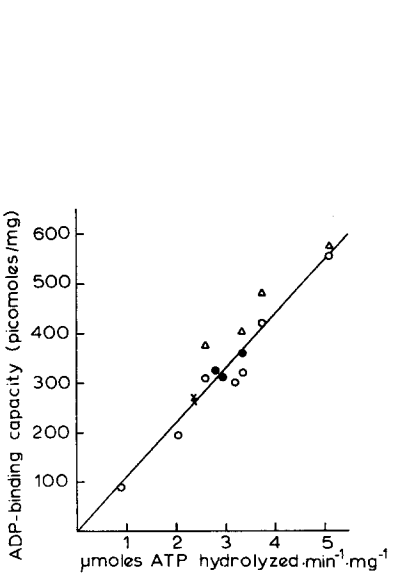
TABLE I

INFLUENCE OF DIMETHYLSULFOXIDE AND DIMETHYLFORMAMIDE ON THE DISSOCIATION CONSTANT OF THE ENZYME–ADP COMPLEX

Experimental conditions	Number of determinations	$K_D \pm$S.D. (μM)
No additions	7	0.946±0.053
10 % dimethylsulfoxide (v/v)	2	0.277±0.070
20 % dimethylsulfoxide (v/v)	4	0.113±0.017
30 % dimethylsulfoxide (v/v)	3	0.092±0.003
20 % dimethylformamide (v/v)	1	0.129

with a mean value of 0.95±0.05 μM for 7 determinations was decreased about 10 fold by 30% dimethylsulfoxide (0.09±0.003 μM). The nucleotide binding capacity, however, is not influenced (Fig. 2). 106 picomoles ADP were maximally bound per unit of $(Na^+ + K^+)$-ATPase under all conditions.

A shift of the pH-optimum of K^+-activated *p*-nitrophenylphosphatase towards more alkaline values was found by Albers and Koval [5]. In order to see whether similar effects can be observed for nucleotide binding, the effect of dimethylsulfoxide on the K_D values for the enzyme–ADP complex at various pH values was investigated.



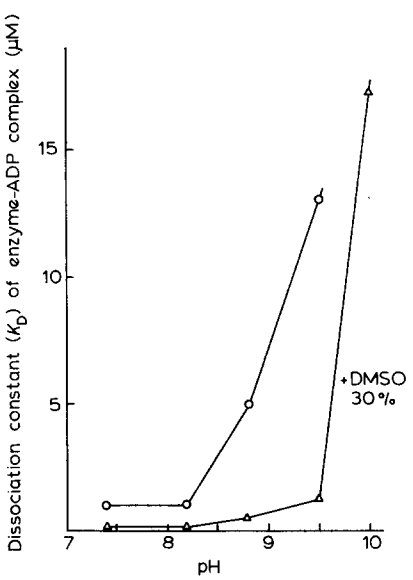


Fig. 2. Comparison of ADP binding capacity of beef brain microsomes and $(Na^+ + K^+)$-ATPase activity at various experimental conditions: control conditions (○), ADP binding capacity in the presence of 5 mM Na^+ (△), binding capacity with 20 % (v/v) dimethylsulfoxide (×), binding capacity with 30 % (v/v) dimethylsulfoxide (●).

Fig. 3. Effect of pH on the dissociation constant (K_D) of the enzyme–ADP complex in the presence and absence of dimethylsulfoxide. Binding in the absence of dimethylsulfoxide, (○–○), binding in the presence of 30 % dimethylsulfoxide, (△–△). 50 mM Tris–buffer was used from pH 7.4–8.8; pH values above pH 8.8 were held constant with 50 mM 2-amino-2-methylpropanol.

TABLE II

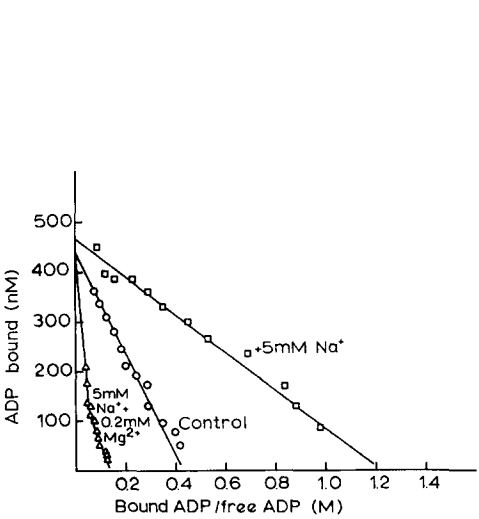
INFLUENCE OF INCREASING Na^+ CONCENTRATIONS ON THE DISSOCIATION CONSTANTS OF THE ENZYME–ADP COMPLEX

Additions	Number of determinations	$K_D \pm$S.D. (μM)
None	7	0.946±0.053
0.5 mM Na^+	1	0.37
1.0 mM Na^+	1	0.33
5.0 mM Na^+	5	0.342±0.075

Fig. 3 shows that ADP binding is independent of pH changes lower than 8.2; it can also be seen that 30% dimethylsulfoxide shifts the sensitivity of the nucleotide receptor for pH changes to more alkaline values.

Na^+ increases [^{14}C]ADP binding [13] by a decrease of the dissociation constant of the enzyme–ADP complex (Table II, Fig. 4), but dimethylsulfoxide does not alter the reactivity of the nucleotide receptor site towards Na^+ (Fig. 5). In the presence as well as in the absence of dimethylsulfoxide, 5 mM NaCl increases the affinity of the nucleotide receptor for ADP about 2 fold.

K^+ decreases nucleotide binding by an increase of the dissociation constant of the enzyme nucleotide complex [13, 16, 21]. This decrease in affinity of the nucleotide for its receptor by K^+ is also seen in the presence of 30% dimethylsulfoxide (Fig. 6). It was found, however, that increasing concentrations of dimethylsulfoxide abolished the action of K^+ on the nucleotide binding site depending on the con-



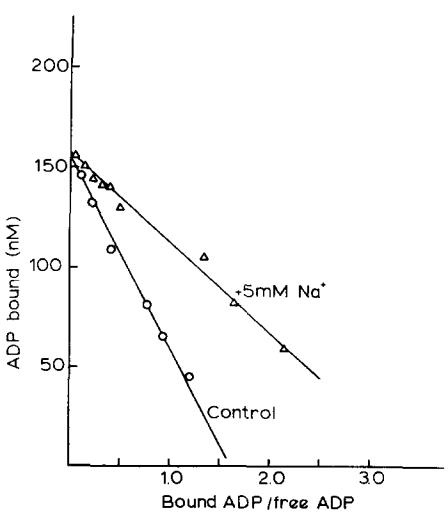


Fig. 4. Scatchard plot of ADP binding to ($Na^+ + K^+$)-ATPase. Effect of 5 mM Na^+ (K_D = 0.38 μM) (□) and 5 mM Na^+ + 0.2 mM Mg^{2+} (△). Control (○) K_D = 1.0 μM.

Fig. 5. Effect of 5 mM Na^+ on the dissociation constant of the enzyme–ADP complex in the presence of 20% (v/v) dimethylsulfoxide. Control (○) K_D = 0.097 μM; K_D in the presence of 5 mM Na^+ (△) = 0.045 μM.

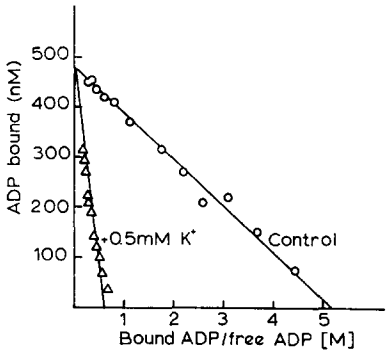


Fig. 6. Effect of 0.5 mM K^+ on ADP binding to $(Na^+ + K^+)$-ATPase in the presence of 30 % (v/v) dimethylsulfoxide (Scatchard plot). $(Na^+ + K^+)$-ATPase activity: 2.8 μmoles · min^{-1} · mg^{-1}, binding in the absence of K^+ (○–○) and in the presence of 0.5 mM K^+ (△). From the slope of the lines the dissociation constants of the enzyme-ADP complex were calculated to be; control (○), $K_D = 0.092\,\mu M$; with 0.5 mM K^+ (△); $K_D = 0.8\,\mu M$.

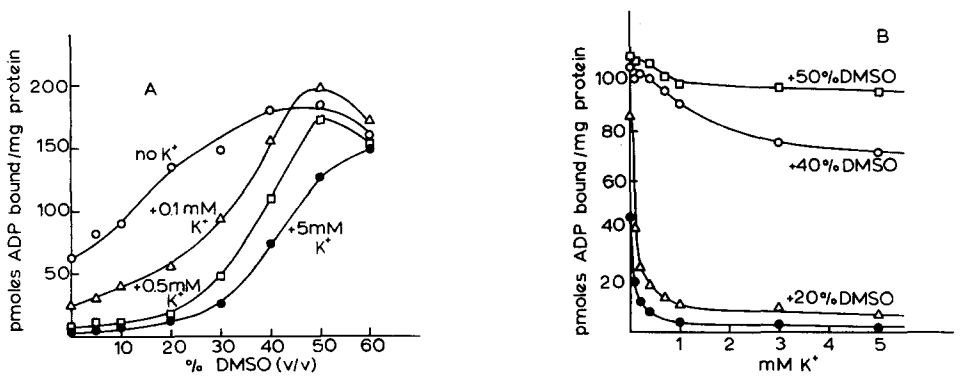


Fig. 7A. Action of increasing concentrations of dimethylsulfoxide on ADP binding to $(Na^+ + K^+)$-ATPase at various K^+ concentrations. Without K^+ (○–○); with 0.1 mM K^+ (△–△); with 0.5 mM K^+ (□–□); with 5 mM K^+ (●–●).

Fig. 7B. Effect of increasing K^+ concentrations on ADP binding to $(Na^+ + K^+)$-ATPase at various concentrations of dimethylsulfoxide: control conditions (●–●), at 20 % dimethylsulfoxide (△–△), at 40 % dimethylsulfoxide (○–○), at 50 % dimethylsulfoxide (□–□).

centrations of both K^+ and dimethylsulfoxide concentrations (Fig. 7).

A decrease of the affinity of $(Na^+ + K^+)$-ATPase for ADP is found with Mg^{2+} and Ca^{2+} [13] but a curved line appears in the Scatchard plot, which indicates a second ADP binding site (Fig. 4). This effect of Mg^{2+} and Ca^{2+} is also observable in the presence of dimethylsulfoxide (Fig. 8). In contrast, however, to the action of dimethylsulfoxide on the K^+-induced change of the affinity of the nucleotide binding site, dimethylsulfoxide does not abolish the effect of Mg^{2+} (Fig. 9).

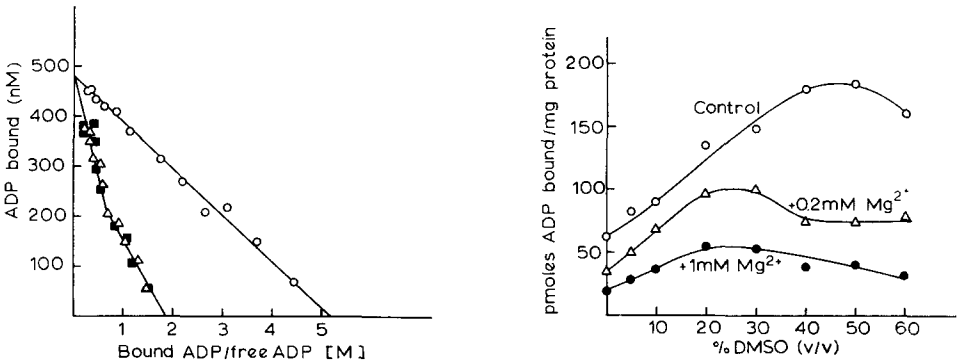


Fig. 8. Effect of Mg^{2+} and Ca^{2+} on ADP binding to $(Na^+ + K^+)$-ATPase in the presence of 30 % (v/v) dimethylsulfoxide (Scatchard plot). ADP binding in the presence of 0.2 mM Mg^{2+} (△–△), 0.2 mM Ca^{2+} (■–■) and in the absence of any additions (○–○). From the slope of the lines the dissociation constants (K_D) of the enzyme–ADP complex were calculated to be: $K_D = 0.092\ \mu M$; with 0.2 mM Mg^{2+} or 0.2 mM Ca^{2+}: high affinity site: $K_D = 0.173\ \mu M$; low affinity site: $K_D = 0.396\ \mu M$.

Fig. 9. Effect of Mg^{2+} on ADP binding to $(Na^+ + K^+)$-ATPase in the presence of increasing concentrations of dimethylsulfoxide. ADP binding in the presence of 1 mM Mg^{2+} (●–●); in the presence of 0.2 mM Mg^{2+} (△–△); in the absence of Mg^{2+} (○–○).

Studies on the rate of phosphorylation and dephosphorylation of $(Na^+ + K^+)$-ATPase

Although dimethylsulfoxide increases nucleotide binding, it inhibits Na^+-dependent ATP–ADP phosphotransferase activity [5]. This enzyme activity probably represents the protein phosphokinase moiety of $(Na^+ + K^+)$-ATPase [22]. As is evident from Fig. 10, the inhibition of this enzyme is caused by slowing down the phosphorylation and dephosphorylation reactions by dimethylsulfoxide. The dephosphorylation reaction was studied in the absence of any additional K^+, since it was shown in previous work that $(Na^+ + K^+)$-ATPase preparations contain sufficient

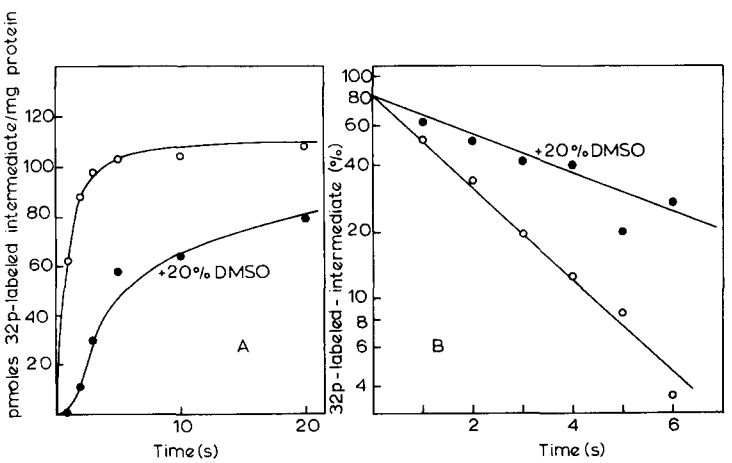


Fig. 10A. Action of 20 % dimethylsulfoxide on the velocity of [^{32}P]phosphoprotein formation.
Fig. 10B. Action of 20 % dimethylsulfoxide on the stability of the [^{32}P]phosphointermediate.

endogeneous K^+ [28]. Because of the low endogeneous K^+ the rate of dephosphorylation was retarded and thus easier to study. The retardation of the dephosphorylation reaction was also observed in the presence of additional K^+. These phosphorylation experiments were performed with enzyme preparations which had been washed extensively with EDTA. This treatment probably converts $(Na^+ + K^+)$-ATPase into the E_1 conformational state [12]. Other conformational states are obtained during active Na^+ transport [12, 18, 22, 23]. In order to check whether the conformational state is important for the inhibition by dimethylsulfoxide, rephosphorylation of the dephosphoenzyme which still contained Rb^+ (a congener of K^+ [1]) in a stable form [18] was studied. The Rb^+-bearing dephosphoenzyme is assumed to be equivalent to the $K^+ \cdot E_2$ conformation state of $(Na^+ + K^+)$-ATPase [18]. It was found that dimethylsulfoxide accelerates the rephosphorylation of the dephosphoenzyme (Fig. 11).

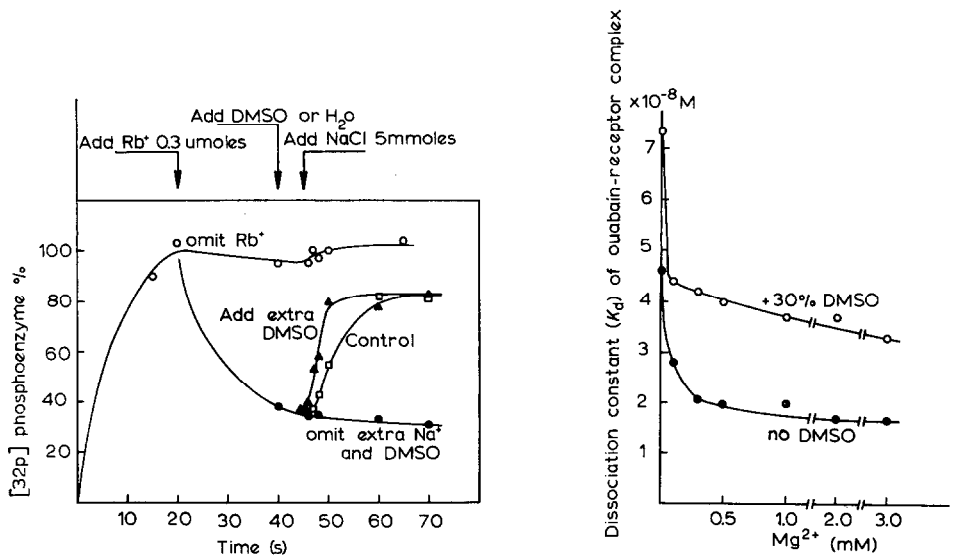


Fig. 11. Comparison of the stability of the dephosphoenzyme produced with Rb^+ in the presence and absence of dimethylsulfoxide. In 1 ml at 0 °C the reaction mixture contained 50 mM Tris–HCl pH 7.4 80 mM NaCl, 0.5 mM $MgCl_2$, 50 μM γ[^{32}P]ATP and 0.8 mg enzyme protein. The reaction was started with enzyme (0.4 ml). At 20 s 0.3 μmole RbCl (0.1 ml) were added (●, ▲, □) or omitted (○). At 40 s 0.2 ml dimethylsulfoxide was added (▲), controls received 0.2 ml H_2O (○, ●, □); at 45 s 0.1 ml 1 M NaCl was added to all samples (○, ▲, □) except (●) which received 0.1 ml H_2O. Final volume of all samples 1.4 ml. The reaction was stopped with acid at the time indicated.

Fig. 12. Effect of dimethylsulfoxide on the dissociation constant of the ouabain–receptor complex at 37 °C. 2-ml reaction volume contained 50 mM imidazole pH 7.25, 3 mM ATP, 100 mM NaCl, increasing concentrations of [^{3}H]ouabain and the $MgCl_2$ concentrations indicated. The dissociation constants were calculated from Scatchard plots.

Studies on ouabain binding

Ouabain is assumed to bind to its receptor when $(Na^+ + K^+)$-ATPase is in the E_2 conformational state [24]. Ouabain binding may proceed via an Na^+-dependent and a Na^+-independent pathway [25]. Because the results in this paper indicate an increase in nucleotide binding by dimethylsulfoxide it was assumed that

dimethylsulfoxide favors the E_1 conformational state. If this assumption were right, the affinity of the ouabain receptor for the cardiac glycoside should be decreased in the presence of dimethylsulfoxide. Indeed, an increase of the dissociation constant of the ouabain receptor complex in the presence of dimethylsulfoxide was found (Fig. 12). It should be mentioned, however, that ouabain binding was not affected very much by dimethylsulfoxide when the binding of cardiac glycoside was studied in the presence of $(Mg^{2+}+P_i)$.

DISCUSSION

The events occurring during $(Na^+ + K^+)$-ATPase catalyzed active transport of sodium and potassium through cellular membranes have been described by eqns 1–5 [18, 23, 32]:

$$Na^+_{in} + ATP + E_1 \overset{Mg^{2+}}{\rightleftharpoons} Na \cdot E_1 \sim P + ADP \quad (1)$$

$$Na \cdot E_1 \sim P \xrightarrow{Mg^{2+}} E_2 \sim P + Na^+_{out} \quad (2)$$

$$K^+_{out} + E_2 \sim P \rightleftharpoons K \cdot E_2 \sim P \quad (3)$$

$$K \cdot E_2 \sim P + H_2O \xrightarrow{K_{in}^+} K \cdot E_2 + P_i \quad (4)$$

$$K \cdot E_2 \underset{Mg^{2+}}{\overset{ATP}{\rightleftharpoons}} E_1 + K^+_{in} \quad (5)$$

The different conformational states can be identified by their ability to bind either ATP or ADP (E_1) or ouabain (E_2) [24]. The E_1 conformational state forming an enzyme–ATP complex ($K_D = 0.12$–$0.22\ \mu M$ [16, 21]) or an ADP–enzyme complex ($K_D = 0.95\ \mu M$) (Table II) is favored by Na^+ (Fig. 4, Table II).

Mg^{2+} reduces the affinity for ADP (Figs 4, 8), probably due to a shift of the E_1 to an E_2 conformation (eqns 2, 5). There also appears to exist a second nucleotide binding site in the presence of Mg^{2+} or Ca^{2+} with or without dimethylsulfoxide (Figs 4, 7) as is evident from the curved line in the Scatchard plot. Binding of ATP to a second nucleotide binding site has been postulated as being responsible for the dissociation of K^+ from the enzyme (eqn. 5) [18, 23]. Binding of Mg^{2+} to the enzyme as measured by the effect of Mg^{2+} on the K_D value of the ADP–enzyme complex appears to be rather tight since it cannot fully be abolished by Na^+ [13], nor does the presence of dimethylsulfoxide alter the reactivity of the nucleotide binding site towards Mg^{2+} (Fig. 9).

In contrast to the effects of Mg^{2+} which may induce the E_2 conformational state [2, 12, 15, 30] it is possible to abolish the reduction of the nucleotide affinity of the nucleotide binding site caused by K^+ (Fig. 6) [13, 16, 21] with increasing Na^+ concentrations [13, 21] and increasing concentrations of dimethylsulfoxide (Fig. 7). It appears that Na^+ shifts the enzyme by competition with K^+ to the E_1 conformational state. Dimethylsulfoxide on the other hand seems to reduce the affinity of the enzyme for K^+ (K^+_{out}?) [6].

K^+ induced a similar effect on the nucleotide receptor at the inner cell surface (Fig. 6) [13, 16, 21] as well as in the ouabain receptor on the outer cell surface [26, 27]. K^+ reduces the affinity of both ligands to their receptor sites. Mg^{2+}, on

the contrary, decreases the affinity of the nucleotide receptor for ADP (and probably ATP) but increases the affinity of the cardiac glycoside receptor for ouabain [26, 27]. If the capability of $(Na^+ + K^+)$-activated ATPase to bind ouabain is a measure of the E_2 conformational state [2, 24, 25], then the K^+-bearing enzyme seems to have a conformation different from the E_2 state.

The effect of Ca^{2+} on the ADP binding site is consistent with an inhibitory action of Ca^{2+} on the Na^+-dependent phosphorylation reaction [31] but inconsistent with the reported Na^+/Ca^{2+} antagonism [31, 32]. If a low affinity for ADP were an indicator of the E_2 conformational state, the decrease of the ADP affinity by Ca^{2+} (Fig. 8) would agree with the effect of Ca^{2+} on the ouabain binding, which is increased in the presence of K^+ by Ca^{2+} [26].

The comparison of the variability of the dissociation constant of the enzyme–ADP complex as a function of the pH value (Fig. 3) with that of the ATP–enzyme complex [21] shows that the terminal γ-phosphate group of ATP has only small effects on the nucleotide receptor interactions compared with changes in the pH above 8. A steep increase of the dissociation constant above pH 8 is also found for the ouabain receptor complex [26]. Since ouabain binds to the outer site and ATP or ADP to the inner site of the cell membrane, it is highly probable that the membrane structure is affected by alkaline medium. The ineffectiveness of alkaline medium in the presence of 30% dimethylsulfoxide (Fig. 3) on the ADP binding site thus seems to reflect a stabilizing effect of this substance on the membrane structure.

Dimethylsulfoxide apparently acts on several parts of the reaction sequence of $(Na^+ + K^+)$-activated ATPase. The increased dissociation constant of the ouabain receptor complex in the presence of dimethylsulfoxide (Fig. 12) is most probably caused by the increase of the concentration of the enzyme–ATP complex at the expense of the concentration of the form of the dephosphoenzyme with which ouabain combines. It has been shown in previous work that there exists a mutual exclusion of ATP and ouabain binding [26, 33]. But, although dimethylsulfoxide increases the affinity of the nucleotide binding site for ATP [6], and ADP (Table I), it decreases the rate of Na^+-dependent phosphorylation (Fig. 10A) (eqn. 1) and the dephosphorylation reaction (Fig. 10B) (eqns 2–4). This finding agrees with the previous observation of an inhibitory action of dimethylsulfoxide on the Na^+-dependent ATP–ADP phosphotransferase activity (eqn. 1) [5]. In accordance with kinetic studies on $(Na^+ + K^+)$-activated ATP hydrolysis [6], dimethylsulfoxide reduced the effect of K^+ on nucleotide binding (Fig. 7). Since it has been shown that the K^+-dependent *p*-nitrophenylphosphatase, which may represent a terminal step in the reaction sequence [2, 7–9], is activated by dimethylsulfoxide (Fig. 1), by an increase of K^+ affinity and the affinity for *p*-nitrophenylphosphate [4–6], it is possible to assume that dimethylsulfoxide does affect the interaction of K^+ with the cation transport system by reducing the affinity of K^+_{out} for its receptor (eqn. 3) and by increasing the dissociation of the potassium–enzyme complex (eqn. 5). The experiment showing an acceleration of the phosphorylation of the K^+-dephosphoenzyme ($K^+ \cdot E_2$) would favor this latter assumption (Fig. 11). If it were so, K^+ should have an effect as an activator of $E_2 \sim P$ hydrolysis, also, in addition to their action as a substrate for the active Na^+, K^+ transport (eqn. 4). This assumption would be in accordance with the conclusions of Albers and Koval [29] who postulate from the effects of Na^+ and K^+ on *p*-nitrophenylphosphatase regulatory and catalytic sites for K^+ and Na^+.

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