Interaction of Solvents with Membranal and Soluble Potassium Ion-Dependent Enzymes

By M. MAYER AND Y. AVI-DOR

Department of Chemistry, Technion-Israel Institute of Technology, Haifa, Israel

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The effects of dimethyl sulphoxide and glycerol on ox brain microsomal $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase (EC 3.6.1.3), K^+ -stimulated *p*-nitrophenyl phosphatase and K^+ -dependent muscle pyruvate kinase (EC 2.7.1.40) were studied. Dimethyl sulphoxide at concentrations below 20% (v/v) was found to stimulate the *p*-nitrophenyl phosphatase and pyruvate kinase by increasing their affinity for K^+ but to inhibit the $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase. The latter enzyme activity was also inhibited by glycerol, which like dimethyl sulphoxide, stimulated the K^+ -activated *p*-nitrophenyl phosphatase at a wide range of concentrations. The solvent effects were promptly reversed by dilution. Similarity was found between glycerol and dimethyl sulphoxide, on one hand, and ATP, on the other, in their stimulatory effect and their ability to increase the ouabain- and oligomycin-sensitivity of the K^+ -stimulated *p*-nitrophenyl phosphatase. However, only the solvents, not the ATP, increased the binding of K^+ by the microsomes. From the above findings it is suggested that solvents may act on K^+ -dependent enzymes by altering the state of solvation of the activating cation as well as by changing the enzyme structure.

Ilani & Tzivoni (1968) showed that soaking of artificial membranes in bromobenzene increases their ion-binding and ion-discriminating abilities. The solvent-induced changes were attributed by the authors to a stronger interaction of the fixed negative charges of the membrane with solvated ions than with hydrated ones, and to the differences in the solvation energy of the various ions. Prompted by the finding described above and the known dependence of a number of soluble and membrane-bound enzymes on ions for their activity (Webb, 1963), we studied the effect of organic solvents on cation-induced enzyme activation.

The present paper deals with the effect of glycerol and DMSO*, both relatively harmless for living cells and enzymes (Ashwood-Smith, 1967; Nash, 1966), on two enzymic reactions that are catalysed by the same membranal enzyme preparation: $\text{Na}^+ + \text{K}^+$ -stimulated ATPase and K^+ -stimulated *p*-nitrophenyl phosphatase. Some data are also presented on the effect of DMSO on a soluble K^+ -activated enzyme, pyruvate kinase.

MATERIALS AND METHODS

Materials. Tris ATP was prepared from the commercial sodium salt by passage through a Dowex 50 (H^+ form)

column and subsequent neutralization with tris. Muscle pyruvate kinase was supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, in an ammonium sulphate suspension and was dialysed for 48 h against 0.1 M-triethanolamine buffer adjusted to pH 7.6 with 0.05 M-HCl.

Enzyme preparation. Ox brain microsomes were prepared and treated as described by Nakao, Tashima, Nagano & Nakao (1965). The same enzyme preparation was used both in the *p*-nitrophenyl phosphatase and ATPase assays.

***p*-Nitrophenyl phosphatase assay.** The basic reaction mixture contained 40 mM-tris buffer adjusted to pH 7.6 with 0.1 M-maleic acid, 5 mM- MgCl_2 , 5 mM-*p*-nitrophenyl phosphate (sodium salt) and the enzyme in a concentration equivalent to 40 μg of protein/ml. The total volume was 1.0 ml and the incubation was at 37°C for 10 min. The reaction was stopped by adding 2.0 ml of 1.0 M-NaOH. The amount of *p*-nitrophenol liberated was determined by measuring the extinction at 400 nm in a Zeiss PMQ II spectrophotometer. Controls were run in which the NaOH was added before the enzyme. The activity was expressed in μmol of *p*-nitrophenol liberated/10 min per mg of protein. The K^+ -dependent increment in activity was denoted as K^+ -stimulated phosphatase.

ATPase assay. The basic reaction mixture contained 20 mM-tris buffer adjusted to pH 7.6 with 0.1 M-HCl, 5 mM- MgCl_2 , 3 mM-tris ATP, 0.5 mM-EDTA and the enzyme in an amount equivalent to 60 μg of protein/ml. The total volume was 1.0 ml and the incubation was at 37°C for 30 min. The reaction was stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid. The amount of P_i liberated

* Abbreviations: DMSO, dimethyl sulphoxide; ATPase, adenosine triphosphatase.

was determined by the method of Fiske & Subbarow (1925). The activity was expressed in μmol of P_i liberated/30 min per mg of protein and the $\text{Na}^+ + \text{K}^+$ -induced increment was denoted as $\text{Na}^+ + \text{K}^+$ -stimulated ATPase.

Pyruvate kinase assay. Pyruvate kinase activity was determined by coupling with lactate dehydrogenase and measuring the rate of NADH disappearance by the method of Bücher & Pfeiderer (1955). The activity was expressed in μmol of NADH oxidized/min per mg of protein.

Determination of protein-bound K^+ . The incubation mixture contained 40 mM-tris buffer adjusted to pH 7.6 with 0.1 M-HCl, 10 mM-KCl, 5 mM-MgCl₂, 5 mM-*p*-nitrophenyl phosphate (sodium salt) and the enzyme in a concentration equivalent to 2 mg of protein/ml. The total volume was 2.0 ml. After incubation at 37°C for 10 min the mixture was centrifuged at 35000g for 40 min in a refrigerated Servall centrifuge. The amount of K^+ in the pellet was determined in an Eppendorf flame photometer after deproteinization with HNO_3 .

Protein determination. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Effect of glycerol and DMSO on $\text{Na}^+ + \text{K}^+$ -stimulated ATPase. Fig. 1 shows the results of an experiment to assay the effect of glycerol and DMSO on the membranal ATPase in presence of an excess of Mg^{2+} and Na^+ and different concentrations of K^+ . In the absence of K^+ the residual activity was not affected by the solvents. In contrast, the K^+ -induced increment in activity was inhibited by both solvents, the extent of inhibition being almost the same in the presence of 2 mM- and 25 mM- K^+ .

The effect of DMSO was also studied under conditions where the K^+ concentration was optimum and that of Na^+ was varied. As shown in Fig. 2, the maximum activity which could be attained in this case was determined by the concentration of DMSO. At Na^+ concentrations that produced less than maximum stimulation the activity of the samples containing DMSO was almost equal to that of the control. The pattern of inhibition described above indicates that the site of action of DMSO is at a step after that stimulated by Na^+ , possibly the one catalysed by K^+ (cf. Glynn, 1968), since if DMSO inhibited the Na^+ -dependent step the curves would be expected to diverge from the control from the origin.

Effect of glycerol and DMSO on K^+ -activated *p*-nitrophenyl phosphatase. The findings described above prompted us to investigate the action of the solvents on K^+ -stimulated *p*-nitrophenylphosphatase, since it is widely held (Bader & Sen, 1966; Albers, 1967; Fujita *et al.* 1966; Izumi, Nagai & Yoshida, 1966; Bader, Post & Bond, 1968) that the K^+ -catalysed step in the hydrolysis of ATP, as well as of

Fig. 1. Effects of glycerol and DMSO on $\text{Na}^+ + \text{K}^+$ -stimulated ATPase. In addition to the components described in the Materials and Methods section, the assay medium contained 140 mM-NaCl. Other additions: ○ and ●, none; △ and ▲, 2 mM-KCl; □ and ■, 25 mM-KCl. ○, △, and □, Glycerol added; ●, ▲ and ■, DMSO added. ATPase activity is expressed in μmol of P_i liberated/30 min per mg of protein.

Fig. 2. Effect of DMSO on $\text{Na}^+ + \text{K}^+$ -stimulated ATPase in the presence of different NaCl concentrations. In addition to the components described in the Materials and Methods section, the assay medium contained 25 mM-KCl and NaCl in the concentration shown. The concentration of DMSO was as follows: ○, none; △, 10% (v/v); □, 20% (v/v); ■, 30% (v/v). Values are corrected for P_i released in the absence of Na^+ but under otherwise identical conditions. ATPase activity is expressed in μmol of P_i liberated/30 min per mg of protein.

p-nitrophenyl phosphate and of some other active phosphate esters, is mediated by the same enzyme entity. As shown in Fig. 3, glycerol up to a concentration of 60% (v/v) increased the activity of K^+ -stimulated phosphatase. DMSO was also stimulatory,

but the plot of activity against concentration showed a maximum with a DMSO concentration of 20% (v/v). Further, Fig. 3 also shows a more pronounced stimulation at 2mM- than at the saturating 25mM-K⁺ concentration, thus indicating a solvent-induced change in the affinity of the enzyme for K⁺. In addition to the change in affinity, some effect of the solvents on the catalytic rate is also apparent from the moderate stimulation of phosphatase activity even at the saturating K⁺-concentration. The above results suggest that the mechanism of the solvent effect is of a rather complicated nature and is not liable to interpretation without a detailed kinetic analysis, which was beyond the scope of the present work.

Fig. 3. Effects of glycerol and DMSO on K⁺-activated *p*-nitrophenyl phosphatase. The reaction mixture and the method of assay were as described in the Materials and Methods section. The notations are the same as in Fig. 1. Phosphatase activity is expressed in μmol of *p*-nitrophenol liberated/10 min per mg of protein.

Reversibility of solvent-induced effect on Na⁺ + K⁺-stimulated ATPase and K⁺-stimulated p-nitrophenyl phosphatase. For testing the reversibility of the solvent-induced effects on Na⁺ + K⁺-stimulated ATPase and K⁺-stimulated *p*-nitrophenyl phosphatase, the enzyme preparation was preincubated with 20% (v/v) DMSO and then diluted tenfold by the assay mixture. In the controls enzyme samples were preincubated in the absence of solvent, and the required amount of DMSO (20% or 2%, v/v) was added only after the dilution. The results in Table 1 show that dilution reversed both the stimulatory and inhibitory effects of the solvents.

Comparison of the effects of DMSO and of ATP on K⁺-affinity and inhibitor-sensitivity of p-nitrophenyl phosphatase. Yoshida, Nagai, Ohasi & Nakagawa (1969) reported stimulation of K⁺-activated *p*-nitrophenyl phosphatase by ATP in the presence of Na⁺. It was therefore decided to compare the ATP-induced effect with the solvent-induced one. From the experiment shown in Fig. 4, it is apparent that ATP (counter-ion Na⁺), like DMSO, exerts its effect by increasing the affinity of the enzyme towards K⁺, but unlike the solvent it has no stimulatory action at higher K⁺ concentrations. Fig. 4 also shows that the effect of ATP is not superimposed on DMSO when both agents are present simultaneously in the assay medium.

The inhibitory effect of ouabain and oligomycin on *p*-nitrophenyl phosphatase was shown to be potentiated by ATP (Fujita *et al.* 1966; Askary & Koyal, 1968). We tested whether DMSO would act similarly to ATP also in this respect. The results summarized in Table 2 show that the DMSO-induced increment in the enzyme activity in the presence of suboptimum concentration of K⁺ (3 mM) was suppressed by ouabain at a concentration that had practically no inhibitory effect in the absence of the solvents. Sensitization by DMSO towards oligomycin was less striking. The ATP-induced enhancement in activity was suppressed by both inhibitors.

Table 1. *Reversibility by dilution of DMSO-induced effects on ATPase and p-nitrophenyl phosphatase*

The enzyme (600 μg of protein/ml) was preincubated at 25°C in a medium containing 20 mM-tris adjusted to pH 7.6 with 0.1 M-HCl, 0.5 mM-EDTA and, when shown, 20% (v/v) DMSO. Preincubation was stopped after 30 min by removing a 0.1 ml sample and mixing it with 0.9 ml of the respective assay medium. Other details were as described in the Materials and Methods section.

Concn. of DMSO (% v/v)		ATPase activity (μmol of P _i released/30 min per mg of protein)		<i>p</i> -Nitrophenyl phosphatase activity (μmol of <i>p</i> -nitrophenol released/10 min per mg of protein)	
In preincubation medium	In assay medium	No NaCl or KCl	140 mM-NaCl +14 mM-KCl	No KCl	4 mM-KCl
None	None	1.89	5.46	0.56	1.15
None	2	1.90	5.49	0.63	1.28
None	20	1.89	2.97	0.62	2.00
20	2	1.89	5.28	0.54	1.30

Fig. 4. Comparison between the effects of DMSO and of ATP on *p*-nitrophenyl phosphatase. The methods of assay were as described in the Materials and Methods section. KCl was added as shown. Other additions: Δ , none; $\bullet$, 20% (v/v) DMSO; $\circ$, 6 μ M-ATP (sodium salt); $\blacktriangle$, 20% (v/v) DMSO + 6 μ M-ATP. Phosphatase activity is expressed in μ mol of *p*-nitrophenol liberated/10min per mg of protein.

Fig. 5. Effects of glycerol and DMSO in the binding of K^+ by the membranal enzyme preparation. The reaction mixture and methods of assay were as described in the Materials and Methods section. The following were added in the concentrations indicated: $\circ$, glycerol; Δ , ATP; $\square$, DMSO.

Table 2. *Effects of ouabain and oligomycin on K^+ -activated *p*-nitrophenyl phosphatase in the presence of DMSO or ATP*

The basic assay medium was supplemented with 3mM-KCl. Other conditions were as described in the Materials and Methods section.

Addition	K ⁺ -stimulated <i>p</i> -nitrophenyl phosphatase activity (μ mol of <i>p</i> -nitrophenol released/10min per mg of protein)		
	No inhibitor	1 μ M-Ouabain	5 μ g of oligomycin
None	0.60	0.58	0.58
20% (v/v) DMSO	0.98	0.59	0.86
60 μ M-ATP	0.84	0.56	0.50

Effect of glycerol, DMSO and ATP on the binding of K^+ by the membranal preparation. In our studies on K^+ -stimulated *p*-nitrophenyl phosphatase the K^+ concentration was equated with that added as potassium chloride to the assay medium. However, in analogy with the findings of Ilani & Tzivoni (1968) in artificial membranes, it seemed feasible that in the presence of solvents a redistribution of the K^+ takes place between the aqueous phase and the membranal particles. Thus the solvent-induced increase in affinity towards K^+ could reflect the increase in K^+ concentration in the vicinity of the enzyme rather than a change in the intrinsic affinity of the enzyme for K^+ . To test this assumption K^+ -binding by the

membrane preparation was determined as a function of solvent concentration. The results in Fig. 5 show that the K^+ -binding capacity of the membrane increased with the solvent concentration. If the solvent concentration is expressed as % (v/v), glycerol promoted the binding of K^+ by the membranal particles more effectively than DMSO. ATP, which, as shown in the previous section, acts in many respects similarly to solvents on K^+ -stimulated *p*-nitrophenyl phosphatase, had no effect on K^+ -binding. Neither the solvents nor ATP changed the inter-particle liquid volume which was determined by the inulin method (Takacs, Matula & MacLeod, 1964) and found to be approx. 50% of the total volume, irrespective of whether solvents or ATP were present or not.

Effect of DMSO on muscle pyruvate kinase activity. We also investigated the effect of DMSO on the K^+ -activated soluble enzyme, muscle pyruvate kinase. In experiments that for the sake of brevity will not be described in detail, it was found that DMSO up to a concentration of 20% (v/v) stimulates the enzyme activity whereas above this concentration the activity declines again. With 20% (v/v) of DMSO in the medium and with the K^+ concentration as the variable parameter it could be shown (Fig. 6) that, as in the case of K^+ -stimulated *p*-nitrophenyl phosphatase, the solvent increased the affinity of the soluble enzyme for K^+ . Pyruvate kinase also shows an obligatory requirement for Mg^{2+} , but no effect of DMSO could be demonstrated on the affinity of the enzyme for Mg^{2+} . Neither could sensitivity of pyruvate kinase to ouabain or oligomycin be induced by addition of DMSO to the reaction medium.

Fig. 6. Effect of DMSO on pyruvate kinase in the presence of different KCl concentrations. The reaction mixture and assay conditions were as described in the Materials and Methods section. Other additions: ○, none; ●, 20% (v/v) DMSO. Results are plotted according to the Lineweaver-Burk method. Pyruvate kinase activity is expressed in μmol of NADH oxidized/min per mg of protein.

DISCUSSION

It has been reported (Bader & Sen, 1966; Fujita *et al.* 1966; Izumi *et al.* 1966; Bader *et al.* 1968) that the K⁺-catalysed step in the hydrolysis of the phosphate group of ATP and of certain phosphate esters, such as *p*-nitrophenyl phosphate and acetyl phosphate, is mediated by the same enzyme. Therefore it is noteworthy that the Na⁺+K⁺-stimulated ATPase was inhibited by a concentration of glycerol or DMSO that enhanced the K⁺-stimulated *p*-nitrophenyl phosphatase activity of brain microsomes. Since the present work shows that the Na⁺-dependent step is not likely to be the one involved in solvent-induced inhibition, it is assumed that the site sensitive to the solvent is located between the Na⁺ and K⁺-dependent steps in the reaction sequence. The existence of more than one intermediate in the overall Na⁺+K⁺-stimulated ATPase reaction has already been suggested by Schoner, Kramer & Seubert (1966) and Nagano *et al.* (1967).

From the results presented here, it is clear that the nature of the solvent action on K⁺-stimulated phosphatase is rather complex since both the maximum velocity of the enzyme activity as well as its affinity for K⁺ were simultaneously changed. This is in contrast with the more simple pattern of ATP-induced activation, where affinity changes alone are involved.

The unequal distribution of K⁺ between the aqueous phase and the membrane particles in presence of DMSO and glycerol may account, at least in part, for the increased affinity of *p*-nitrophenyl phosphatase towards K⁺ observed in the presence of the solvent. The increase in binding affinity is probably connected with solvation of K⁺ rather than

with an effect on the site of binding in the membrane, in analogy with the effect of solvents on cation-binding by artificial membranes (cf. Ilani & Tzivoni, 1968). Although the effect of solvents on the binding of K⁺ by the soluble enzyme pyruvate kinase has not been studied, solvation of the cation would be expected also in this case to cause a stronger interaction between the binding site and K⁺, and as a corollary, to increase affinity, as found experimentally.

The increase in the sensitivity of *p*-nitrophenyl phosphatase towards oligomycin and ouabain inhibition in the presence of solvents is, however, in all likelihood not related to solvation of K⁺. Increased binding of K⁺ would antagonize rather than enhance the effect of ouabain (cf. Dunham & Glynn, 1961; Charnock & Post, 1963), and the effect of oligomycin is known to be independent of the prevailing K⁺ concentration (Ahmed, Judah & Scholefield, 1966). Thus the possibility exists that some of the solvent effects are due to conformational changes induced in the enzyme protein, as suggested by Yoshida *et al.* (1969) for certain ATP-induced changes in the same microsomal enzyme preparation. It is noteworthy in this context that the present work shows that the ATP-dependent changes are not accompanied by an increased K⁺-binding capacity of the membrane preparation.

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