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DIFFERENTIAL MODIFICATION OF THE $(Na^+ + K^+)$-DEPENDENT ATPase BY DIMETHYLSULFOXIDE

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SUMMARY

1. Dimethylsulfoxide reversibly inhibited the $(Na^+ + K^+)$-dependent ATPase activity of a brain microsomal enzyme preparation, but stimulated the associated K^+-dependent phosphatase activity; this disparate effect was not caused by two other lipophilic agents, Lubrol-W and propanol.

2. For the ATPase dimethylsulfoxide reduced the concentration for half-maximal activation, $K_{0,5}$, for NaCl, but increased it for KCl; in both cases V was decreased. Since both V and K_m for ATP were also decreased in parallel, it thus appeared that dimethylsulfoxide was an uncompetitive inhibitor to ATP and Na^+, perhaps acting to modify a stage following their reaction with the enzyme, and thereby altering the apparent affinity for K^+.

3. With the phosphatase, however, dimethylsulfoxide reduced $K_{0,5}$ for KCl and increased V. But in the presence of CTP and NaCl, which have been shown to modify the phosphatase reaction to resemble more closely the ATPase, dimethylsulfoxide then increased $K_{0,5}$ for KCl although V was still increased. The stimulation of the phosphatase could be accounted for by a decrease in K_m for the substrate, nitrophenylphosphate.

4. The effects on apparent affinity for K^+ thus represent an action of dimethylsulfoxide dependent both on the route of substrate entry (Na^+-dependent for the ATPase, K^+-dependent for the phosphatase) and the reaction history (prior interaction with nucleotide *plus* Na^+, or not). If dimethylsulfoxide acts by selecting or modifying intermediary reaction conformations (or their analogues), then the variable cation affinity shown for such intermediate stages may bear on the cyclical changes in affinity proposed to accompany the hydrolytic process.

INTRODUCTION

Dimethylsulfoxide has recently been shown[1] to inhibit the $(Na^+ + K^+)$-dependent ATPase but to stimulate the K^+-dependent phosphatase that appears to reflect the terminal K^+-dependent hydrolytic process of the ATPase[2-4]. Although the mechanism of this stimulation/inhibition was unclear, Mayer and Avi-Dor[1] showed an

increase in apparent affinity of the phosphatase for K^+, and consequently proposed that dimethylsulfoxide caused a generalized increase in affinity of enzymatic systems for K^+. Other agents also display disparate effects on the ATPase and phosphatase activities, and kinetic studies of cation activation have suggested certain modes of interaction in relation to the reaction mechanism[5,6]. It therefore seemed of interest to extend these approaches and to examine in this vein the effects of dimethylsulfoxide on cation activation of both the ATPase and the phosphatase. Although these experiments confirm an increased apparent affinity of the phosphatase for K^+, they show a decreased affinity of the ATPase for K^+. Moreover, in the presence of certain modifiers the affinity of the phosphatase for K^+ was decreased by dimethylsulfoxide, in accord with its effects on the ATPase. These data may then be incorporated into considerations both of the relationship between phosphatase and the ATPase and of the overall reaction process.

METHODS

The $(Na^+ + K^+)$-dependent ATPase was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described[7].

$(Na^+ + K^+)$-dependent ATPase activity was measured in terms of the production of P_i, as previously described[7]. The standard medium contained 50 mM histidine–HCl (pH 7.8 with Tris), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, 10 mM KCl, and the enzyme preparation (0.1 mg protein/ml). Incubation was for 4–8 min at 37 °C; activity was linear with time during these periods. Activity in the absence of Na^+ and K^+ ("Mg^{2+}-ATPase") was measured concurrently; such activity averaged only a few percent of the $(Na^+ + K^+)$-dependent ATPase activity[7], and was subtracted from the total activity in the presence of Na^+ and K^+ to give the $(Na^+ + K^+)$-dependent activity. Because of variations in the absolute activity of different enzyme preparations, enzyme velocities are expressed relative to the $(Na^+ + K^+)$-dependent ATPase activity of a concurrent control incubation in the standard medium, defined as 1.0. This control activity averaged 2.6 μmoles P_i liberated/mg protein per min.

K^+-dependent phosphatase activity was measured in terms of the production of *p*-nitrophenol after incubation with *p*-nitrophenylphosphate, as previously described[4]. The standard medium contained 50 mM histidine (pH 7.8), 3 mM $MgCl_2$, 3 mM nitrophenylphosphate (as the Tris salt), 20 mM KCl, and the enzyme preparation (0.1 mg protein per ml). Incubation was for 8–15 min at 37 °C; activity in the absence of added KCl was measured concurrently; such activity averaged only a few percent of the K^+-dependent phosphatase activity under optimal conditions[4], and was subtracted from the total activity in the presence of KCl to give the K^+-dependent activity. As with the ATPase, velocities are expressed relative to the K^+-dependent phosphatase activity of a concurrent control incubation in the standard medium, defined as 1.0. This control activity averaged 0.17 μmoles nitrophenol liberated/mg protein per min.

ATP, CTP, and nitrophenylphosphate were purchased from Sigma Chemical Co. as the sodium salts, and converted to the Tris salts. All solutions were made in water that had been redistilled from an all-glass still. Protein was measured by the biuret method, using bovine serum albumin as a standard.

Experimental points are the average of five or more experiments performed in duplicate. Values from the Hill plots of n and $K_{0.5}$ were calculated from the equations for the straight lines obtained by the method of least squares, with standard deviations calculated as previously described[7].

RESULTS

Inhibition of the ATPase by dimethylsulfoxide

Dimethylsulfoxide inhibited the $(Na^+ + K^+)$-dependent ATPase at concentrations of 5–40 % (v/v), as shown also by Mayer and Avi-Dor[1], and a concentration of 10 % dimethylsulfoxide was selected for further experiments as a compromise between magnitude of inhibition and influence on solute concentrations. Inhibition was present after the shortest feasible incubation times (1.5 min), and remained constant at this level throughout the incubation periods used. In addition, the inhibition due to dimethylsulfoxide was reversible; inhibition of an enzyme preparation treated with dimethylsulfoxide was removed by washing (centrifugation and resuspension).

For comparison with dimethylsulfoxide several other lipophilic agents were tested on the ATPase. Lubrol-W and 1-propanol were selected for further study because of the immediate onset of inhibition, which remained constant throughout the incubation period. By contrast, inhibition by Triton X-100 and Tween-20 was slow in onset and progressed during incubation, making these agents unsuitable for kinetic studies.

Effects of dimethylsulfoxide on cation activation of the ATPase

At a constant concentration of KCl, 10 mM, the velocity–activator curve for Na^+ was sigmoidal, with a Lineweaver–Burk plot concave upward and a Hill plot with slope $n > 1.0$ (Fig. 1), as previously shown[7]. Dimethylsulfoxide altered this

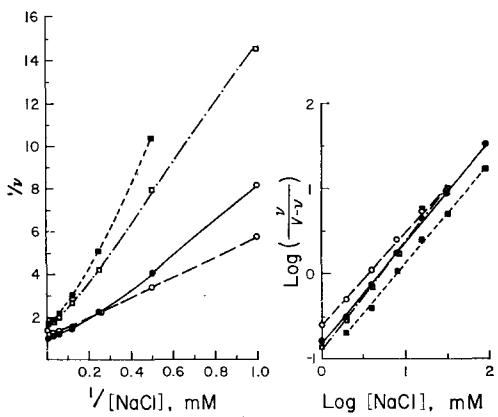

Fig. 1. Na^+ activation of the $(Na^+ + K^+)$-dependent ATPase. The enzyme preparation was incubated at 37 °C in the standard medium (see Methods) but with the concentrations of NaCl shown (●). Parallel experiments were performed in the presence of 10% (v/v) dimethylsulfoxide (○), 2% (v/v) 1-propanol (□), and 0.3% (w/v) Lubrol-W (■). Data are presented in the left hand panel in the form of a Lineweaver–Burk plot, and in the right hand panel in the form of a Hill plot with the straight lines drawn by the method of least squares. Kinetic parameters for control, dimethylsulfoxide, propanol, and Lubrol incubations are, respectively: V, 1.03, 0.80, 0.58, and 0.64 relative units; $K_{0.5}$, 5.0, 3.6, 5.0 and 8.2 mM; and n, 1.19, 1.06, 1.29 and 1.20.

TABLE I

Na^+ ACTIVATION OF THE $(Na^+ + K^+)$-DEPENDENT ATPase

Experiments were performed as in Fig. 1, either with a constant concentration of KCl (10 mM) or varying NaCl and KCl together at a constant ratio of 9:1, in the presence and absence of 10% dimethylsulfoxide. Data are presented ± S.D.

Conditions	*Additions*	*Kinetic parameters for* Na^+		
		V	$K_{0.5}$ (*mM*)	n
With 10 mM KCl	None	1.03 ± 0.02	5.0 ± 0.6	1.19 ± 0.04
	Dimethylsulfoxide	0.80 ± 0.02	3.6 ± 0.6	1.06 ± 0.04
With [NaCl]:[KCl] = 9:1	None	1.10 ± 0.04	7.2 ± 0.9	0.98 ± 0.05
	Dimethylsulfoxide	0.89 ± 0.02	12.1 ± 1.6	0.69 ± 0.03

TABLE II

K^+ ACTIVATION OF THE $(Na^+ + K^+)$-DEPENDENT ATPase

Experiments were performed as in Fig. 2, either with a constant concentration of NaCl (90 or 5 mM) or varying NaCl and KCl together at a constant ratio of 9:1, in the presence and absence of 10% dimethylsulfoxide. Data are presented ± S.D.

Conditions	*Additions*	*Kinetic parameters for* K^+		
		V	$K_{0.5}$ (*mM*)	n
With 90 mM NaCl	None	1.03 ± 0.02	0.81 ± 0.12	1.37 ± 0.05
	Dimethylsulfoxide	0.80 ± 0.02	1.30 ± 0.17	1.11 ± 0.06
With 5 mM NaCl	None	0.63 ± 0.01	0.14 ± 0.01	1.18 ± 0.05
	Dimethylsulfoxide	0.50 ± 0.01	0.19 ± 0.02	1.02 ± 0.04
With [NaCl]:[KCl] = 9:1	None	1.10 ± 0.04	0.80 ± 0.10	0.98 ± 0.04
	Dimethylsulfoxide	0.89 ± 0.02	1.34 ± 0.17	0.69 ± 0.03

response, decreasing V, n, and $K_{0.5}$, the concentration for half-maximal activation (Table I). By contrast, inhibitory concentrations of Lubrol and propanol either had no effect on $K_{0.5}$ and n, or increased them (Fig. 1).

On the other hand, when Na^+ and K^+ were varied together at a constant ratio of 9:1, dimethylsulfoxide increased the $K_{0.5}$ for Na^+ (Table I). However, this result may reflect the preponderant effect of dimethylsulfoxide on the $K_{0.5}$ for K^+ (see below), to which the Na^+ concentration in these experiments is tied at a constant ratio.

The response to K^+, in the presence of 90 mM NaCl, was also sigmoidal, with a Lineweaver–Burk plot concave upward and a Hill plot with slope $n > 1.0$ (Fig. 2). Dimethylsulfoxide again decreased V and n, but here it increased $K_{0.5}$ (Table II). This effect of dimethylsulfoxide was also seen both at a constant Na^+:K^+ ratio of 9:1 (see above), and in the presence of a constant low concentration of NaCl, 5 mM (Table II), where competitive interactions between Na^+ and K^+ might be expected to be minimized[8].

Lubrol and propanol similarly increased $K_{0.5}$ for K^+ although the effect on n was less marked (Fig. 2).

Effect of dimethylsulfoxide on cation activation of the phosphatase

For the K^+-dependent phosphatase activity, measured with nitrophenylphosphate, the response to K^+ was also sigmoidal, although the $K_{0.5}$ for K^+ was considerably higher than for the ATPase (Fig. 3; Table III), as previously shown[4,8]. However, concentrations of dimethylsulfoxide that inhibited the ATPase stimulated the phosphatase, and with 10% dimethylsulfoxide V was increased whereas $K_{0.5}$ for K^+ was decreased (Fig. 3; Table III); as with the ATPase n was decreased.

On the other hand, the effects of Lubrol and propanol on these kinetic parameters were similar to their effects on those of the ATPase: V was decreased, $K_{0.5}$ for K^+ was increased, and n was unchanged or diminished (Fig. 3).

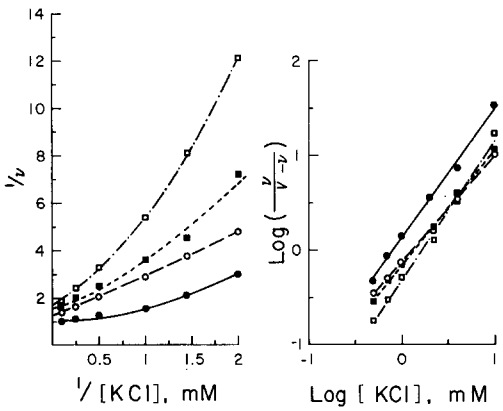

Fig. 2. K^+ activation of the ($Na^+ + K^+$)-dependent ATPase. The enzyme preparation was incubated at 37 °C in the standard medium (see Methods) but with the concentrations of KCl shown (●). Parallel experiments were performed in the presence of 10% dimethylsulfoxide (○), 2% propanol (□), and 0.3% Lubrol (■). Data are presented as in Fig. 1. Kinetic parameters for control, dimethylsulfoxide, propanol, and Lubrol incubations are, respectively: V, 1.03, 0.80, 0.59 and 0.66 relative units; $K_{0.5}$, 0.81, 1.30, 1.65 and 1.29 mM; and n, 1.37, 1.11, 1.48 and 1.29.

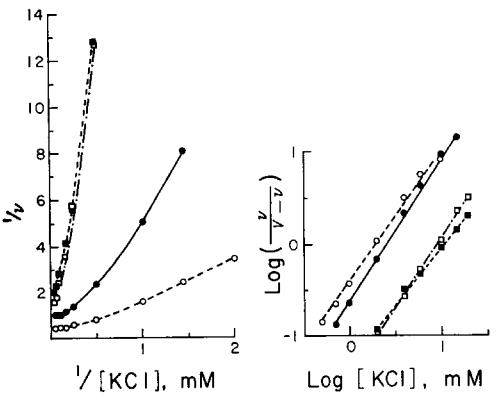

Fig. 3. K^+ activation of the K^+-dependent phosphatase. The enzyme preparation was incubated at 37 °C in the standard medium (see Methods) but with the concentrations of KCl shown (●). Parallel experiments were performed in the presence of 10% dimethylsulfoxide (○), 2% propanol (□), and 0.3% Lubrol (■). Data are presented as in Fig. 1. Kinetic parameters for control, dimethylsulfoxide, propanol, and Lubrol incubations are, respectively: V, 1.06, 2.31, 0.82 and 0.75 relative units; $K_{0.5}$, 2.6, 2.1, 9.2 and 11.2 mM; n, 1.50, 1.26, 1.48 and 1.21.

TABLE III

K^+ ACTIVATION OF THE K^+-DEPENDENT PHOSPHATASE

Experiments were performed as in Fig. 3, either in the absence of NaCl, with 10 mM NaCl, or with 10 mM NaCl *plus* 0.3 mM CTP, in the presence and absence of 10% dimethylsulfoxide. Data are presented ± S.D.

Conditions	*Additions*	*Kinetic parameters for* K^+		
		V	$K_{0.5}$ (*mM*)	n
No NaCl	None	1.06 ± 0.03	2.6 ± 0.3	1.50 ± 0.06
	Dimethylsulfoxide	2.31 ± 0.06	2.1 ± 0.2	1.26 ± 0.05
With 10 mM NaCl	None	1.05 ± 0.02	3.3 ± 0.5	1.00 ± 0.06
	Dimethylsulfoxide	2.34 ± 0.05	0.8 ± 0.2	0.87 ± 0.06
With 10 mM NaCl *plus* 0.3 mM CTP	None	0.86 ± 0.02	0.09 ± 0.01	0.94 ± 0.04
	Dimethylsulfoxide	1.88 ± 0.04	0.21 ± 0.05	0.93 ± 0.05

To determine if this difference in effect of dimethylsulfoxide on the kinetic response to K^+ of the two enzymatic activities was due to the presence of Na^+ in the one case, phosphatase activity was also measured with 10 mM NaCl, a concentration between those tested with the ATPase (because of inhibition of the phosphatase much higher concentrations of NaCl are precluded[4]). Again, dimethylsulfoxide increased V but decreased $K_{0.5}$, in direct contrast to its effects on the ATPase.

In the presence of both low concentrations of certain nucleotides and Na^+, the K^+-dependent phosphatase activity is modified to a form that may more closely resemble the ATPase[3,4,9]: the $K_{0.5}$ for K^+ is markedly reduced[4,9] and sensitivity to oligomycin is greatly increased[3,6]. Under these circumstances, in the presence of 0.3 mM CTP *plus* 10 mM NaCl, dimethylsulfoxide still increased V markedly, but now it also increased $K_{0.5}$ for K^+ (Table III), as it did for the ATPase. Moreover, under these conditions dimethylsulfoxide increased the apparent affinity for Na^+, the $K_{0.5}$ for NaCl decreasing from 3.1 mM in the absence of dimethylsulfoxide to 1.8 mM in its presence, in close analogy to the effects of dimethylsulfoxide on the apparent affinity of the ATPase for Na^+.

As with the ATPase activity, the effects of dimethylsulfoxide on the phosphatase activity could be reversed by washing.

Effects of dimethylsulfoxide on substrate utilization

Since the effects of dimethylsulfoxide on cation activation cannot explain the disparate stimulation of the phosphatase and inhibition of the ATPase, the effect of dimethylsulfoxide on the kinetics of substrate utilization was examined. For the ATPase, dimethylsulfoxide was an uncompetitive inhibitor, *i.e.*, both V and K_m were decreased (Fig. 4). For the phosphatase, however, dimethylsulfoxide was a "competitive" activator, *i.e.* V was unchanged but K_m was decreased (Fig. 4); because of marked substrate inhibition of the phosphatase[4] saturating levels of nitrophenylphosphate are not practical in standard assay media.

Effects of temperature on response to dimethylsulfoxide

Comparison of ATPase activity at 30 and 20 °C revealed a Q_{10} of 3.1, consistent with an energy of activation of 20 kcal (ref. 7). In the presence of dimethylsulfoxide

the Q_{10} diminished (Table IV). For the phosphatase activity the Q_{10} was considerably lower, 1.9, comparable to an energy of activation around 11 kcal. In this case dimethylsulfoxide increased the Q_{10} slightly (Table IV). The significant observation, however, is that at 20 °C the effect of dimethylsulfoxide (either inhibition of ATPase or stimulation of phosphatase) was less than at 30 °C.

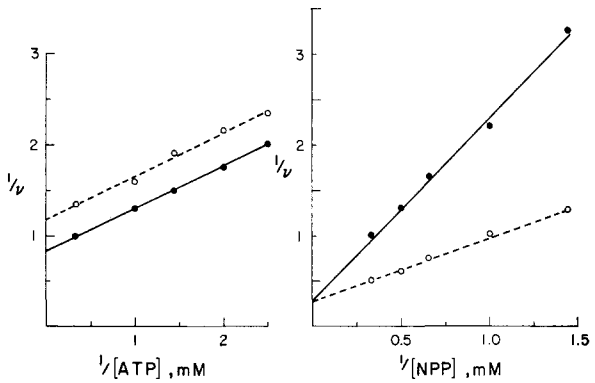

Fig. 4. Effects of substrate on the $(Na^+ + K^+)$-dependent ATPase and K^+-dependent phosphatase activities. For studying ATPase activity the enzyme preparation was incubated at 37 °C in the standard medium (see Methods) but with ATPase and $MgCl_2$ (at a 1:1 molar ratio) at the concentration shown, in the absence (●) and presence (○) of 10% dimethylsulfoxide. These data are presented in the left hand panel in the form of a Lineweaver–Burk plot with the lines drawn by the method of least squares. For the phosphatase activity the enzyme was incubated at 37 °C in the standard medium (see Methods) but with nitrophenylphosphate (NPP) at the concentrations shown, in the absence (●) and presence (○) of 10% dimethylsulfoxide. These data are presented in the right hand panel.

TABLE IV

EFFECTS OF TEMPERATURE ON THE ACTION OF DIMETHYLSULFOXIDE

Enzymatic activity was measured in the standard media (see Methods) in the presence and absence of 10% dimethylsulfoxide, at the temperature indicated.

Enzymatic activity	*Additions*	*Relative velocity*		Q_{10}
		30 °C	*20 °C*	
ATPase	None	1.00	0.32	3.1
	Dimethylsulfoxide	0.72	0.29	2.5
Phosphatase	None	1.00	0.53	1.9
	Dimethylsulfoxide	2.10	0.94	2.2

DISCUSSION

Certain characteristics of the activation of the $(Na^+ + K^+)$-dependent ATPase by monovalent cations have been described, yet the detailed relationship of these interactions to the overall enzymatic process, as well as to the physiological mechanism for cation transport, is still unclear (for recent reviews see Skou[10] and Schoner[11]). For example, it is uncertain whether the same sites bind alternately Na^+ and K^+ or

whether each cation has its own class of sites, and although most schemes for cation transport invoke a change in cation affinity during the reaction cycle, demonstrations of such changes with the ATPase are lacking. One approach to these issues is to use inhibitors that influence various partial reactions of the ATPase, in attempts both to compare the properties of these reactions and to place them within the total reaction sequence. In this context dimethylsulfoxide is a useful reagent, despite ignorance about the molecular mode by which such substances influence protein structure, for it caused a reversible, temperature-dependent inhibition of the ATPase activity, but a stimulation of the associated K^+-dependent phosphatase activity, a pattern not shared by two other lypophilic agents, Lubrol and propanol.

For the ATPase the salient effects of dimethylsulfoxide were: (i) parallel decreases in V and K_m (or $K_{0.5}$) for both ATP and Na^+; (ii) for K^+ a decrease in V but an increase in $K_{0.5}$; and (iii) for each cation a decrease in n, a measure of the cooperativity between activating sites. This pattern of uncompetitive inhibition toward both ATP and Na^+ is consistent with dimethylsulfoxide influencing reaction stages following the interaction of the enzyme with Na^+ and ATP (*cf.* ref. 12), as proposed previously by Mayer and Avi-Dor[1]. An alternative explanation for this pattern of inhibition, that dimethylsulfoxide can react only with the immediate product of enzyme, ATP, and Na^+, the acyl phosphate form of the enzyme, is unlikely both because of the chemical nature of this inhibitor and because dimethylsulfoxide also influenced the phosphatase reaction in which a Na^+-dependent phosphorylation is not an obligatory step[13]. It would seem, therefore, that dimethylsulfoxide modifies the enzyme structure so that the reaction progress is interrupted at a step subsequent to the initial phosphorylation, perhaps by stabilizing an intermediary stage (or favoring a close structural analogue of such a stage) in the sequence of protein conformations associated with the reaction. This formulation is supported by consideration of the effects of dimethylsulfoxide on K^+ kinetics, since K^+ is required for the subsequent liberation of phosphate from the enzyme[10,11] and dimethylsulfoxide decreased the apparent affinity for K^+.

To examine these processes further, the effects of dimethylsulfoxide on the K^+-dependent phosphatase activity may be considered. This enzymatic process apparently reflects a direct entry of the phosphatase substrate to the hydrolytic site, bypassing the initial Na^+-dependent phosphorylation of the ATPase[13,14]. The stimulation of the phosphatase by dimethylsulfoxide bears on this point, for it reflects an increase in affinity for the substrate, nitrophenylphosphate: V is unchanged but K_m is decreased. Thus dimethylsulfoxide apparently facilitates entry of an artificial substrate, nitrophenylphosphate, to the hydrolytic site, while hindering hydrolysis of the normal substrate, the acyl phosphate intermediate, perhaps by affecting a K^+-dependent migration of this intermediate to the hydrolytic site[15]. Nitrophenylphosphate, therefore, appears to avoid a rate-limiting step of the overall ATPase reaction.

The phosphatase reaction also differs from the ATPase in having a considerably larger $K_{0.5}$ for K^+, and this $K_{0.5}$ is markedly reduced in the presence of Na^+ and certain nucleotides[4,9], conditions under which the enzyme may form an acyl phosphate intermediate. Correspondingly, dimethylsulfoxide affected the $K_{0.5}$ for K^+ under these two conditions in opposite ways: it decreased the $K_{0.5}$ for K^+ in the absence of nucleotides, but increased it in the presence of Na^+ and CTP just as it did for the ATPase reaction. Thus it would seem that dimethylsulfoxide affected the apparent

affinity of K^+ in a manner reflecting the route of substrate entry and the preceding conformations of the enzyme. For both the ATPase and the phosphatase in the presence of Na^+ and a nucleotide a high affinity for K^+ was reduced by dimethylsulfoxide, whereas for the ordinary phosphatase reaction a low affinity for K^+ was increased by dimethylsulfoxide:

	Affinity for K^+			
	Low	*High*	*Intermediate*	*Low*
ATPase	$E \xrightarrow{ATP} E\text{–}P \longrightarrow$		$[E'\text{–}P] \longrightarrow$	$E + P_i$
Phosphatase	$E \xrightarrow{NPP^*}$		$[E'\text{–}P^*] \longrightarrow$	$E + P_i{}^*$
Phosphatase with nucleotide	$E \xrightarrow{CTP} E\text{–}P \xrightarrow{NPP^*}$		$[E'\text{–}P^*] \longrightarrow$	$E + P_i{}^*$

where NPP represents nitrophenylphosphate; the addition sequence for cations is unspecified. In this scheme dimethylsulfoxide would favor, by influencing the protein structure, an enzyme conformation similar to $[E'\text{–}P]$, a form common to both the ATPase and phosphatase reaction sequences, representing the enzyme–phosphate complex at the hydrolytic site and differing from the initial Na^+-dependent phosphorylated complex[14]. Consequently, dimethylsulfoxide would modify the apparent affinity for K^+ to an intermediate level, from either a high or low affinity form. Insofar as dimethylsulfoxide may be acting to modify the reaction sequence at a stage where the affinity for K^+ may be varying, to reduce the affinity for K^+ in one mode of substrate entry and to increase affinity in another, then such a formulation, albeit tentative, offers support for numerous suggestions linking cation transport to cyclical changes in cation affinity.

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