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SOLVENT EFFECTS ON OUABAIN BINDING TO THE (Na^+,K^+)-ATPase OF RAT BRAIN

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The effects of the solvents deuterated water (^2H_2O) and dimethyl sulfoxide (Me_2SO) on [^{3}H]ouabain binding to (Na^+,K^+)-ATPase under different ligand conditions were examined. These solvents inhibited the type I ouabain binding to the enzyme (i.e., in the presence of $Mg^{2+} + ATP + Na^+$). In contrast, both solvents stimulated type II (i.e., $Mg^{2+} + P_i$-, Mg^{2+}-, or Mn^{2+}-dependent) binding of the drug. The solvent effects were not due to pH changes in the reaction. However, pH did influence ouabain binding in a differential manner, depending on the ligands present. For example, changes in pH from 7.05 to 7.86 caused a drop in the rate of binding by about 15% in the presence of $Mg^{2+} + Na^+ + ATP$, 75% in the $Mg^{2+} + P_i$ system, and in the presence of Mn^{2+} an increase by 24% under similar conditions. Inhibitory or stimulatory effects of solvents were modified as various ligands, and their order of addition, were altered. Thus, ^2H_2O inhibition of type I ouabain binding was dependent on Na^+ concentration in the reaction and was reduced as Na^+ was elevated. Contact of the enzyme with Me_2SO, prior to ligands for type I binding, resulted in a greater inhibition of ouabain binding than that when enzyme was exposed to $Na^+ + ATP$ first and then to Me_2SO. Likewise, the stimulation of type II binding was greater when appropriate ligands acted on enzyme prior to addition of the solvent. Since Me_2SO and ^2H_2O inhibit type I ouabain binding, it is proposed that this reaction is favored under conditions which promote loss of H_2O, and E_1 enzyme conformation; the stimulation of type II ouabain binding in the presence of the solvents suggests that this type of binding is favored under conditions which promote the presence of H_2O at the active enzyme center and E_2 enzyme conformation. This postulation of a role of H_2O in modulating enzyme conformations and ouabain interaction with them is in concordance with previous observations.

Introduction

(Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) represents the enzymic basis of cation transport across the cell membrane, and is also a putative receptor for cardiac glycosides [1–6]. We have previously proposed that H_2O may play a regulatory role in the active center of the (Na^+, K^+)-ATPase [7–10]. This was based on the observation that removal of H_2O from the active center promoted the E_1 form, whereas its presence promoted the E_2 form of the enzyme. It appeared that Na^+-dependent phosphoenzyme formation was promoted by removal of H_2O. The converse, i.e. presence of H_2O was found to favor both the interaction of K^+ and the hydrolysis of the phosphoenzyme intermediate.

Speculating that H_2O at the active center of (Na^+,K^+)-ATPase may also influence the interac-

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tion of ouabain with the enzyme, we examined the effects of solvents such as ^2H_2O (deuterated water) and Me_2SO (dimethyl sulfoxide) which can replace bound H_2O, and as such, would be expected to stimulate or inhibit, respectively, reactions requiring or not requiring the presence of H_2O at the active center (see, for example, Refs. 7–10). We have demonstrated that these solvents inhibit the type I (i.e., $Mg^{2+}+ATP+Na^+$-stimulated) but markedly stimulate the type II (i.e., $Mg^{2+}+P_i$-, or Mg^{2+}-, or Mn^{2+}-stimulated) binding of ouabain to the enzyme (definition of types of ouabain binding is essentially as in Ref. 11). The results are interpreted in terms of a role of H_2O on the enzyme conformations and its consequences for the ouabain interaction with them. Preliminary accounts of this work have been given [12,13]. A study of the effects of these solvents on the dissociation of ouabain from its receptor will be presented in a subsequent paper.

Experimental procedures

Materials

[^{3}H]Ouabain was obtained from New England Nuclear. All other details of various materials used in these experiments have been given previously [8,9].

Methods

Preparation of (Na^+,K^+)-ATPase. The details of preparation and properties of rat brain membrane (Na^+,K^+)-ATPase are the same as given previously [13,14]. The specific activity of the Na^+,K^+-stimulated enzyme was generally 125 μmol of P_i/mg of protein per h, while the basic Mg^{2+}-stimulated activity was about 5–10% of the total ($Mg^{2+}+Na^++K^+$)-dependent ATPase.

Procedure for [^{3}H]ouabain binding. The procedures for measuring specific [^{3}H]ouabain binding were the same as described by Wallick and Schwartz [16]. Ligand conditions (or other specific variations) are given under appropriate legends. When present, the solvents ^2H_2O and Me_2SO were at concentrations of 85% and 30% (v/v), respectively. The effects produced at these concentrations are freely reversible.

Results

Effects of ^2H_2O on [^{3}H]ouabain binding under different ligand conditions

It has been suggested that types I and II ouabain binding to (Na^+,K^+)-ATPase occurs through different pathways involving different conformations of the enzyme [11,17–25]. It has not been clearly shown which conformations may be associated

TABLE I

ANTAGONISM BETWEEN Na^+ AND ^2H_2O ON [^{3}H]OUABAIN BINDING IN THE PRESENCE OF $Mg^{2+}+ATP+Na^+$

The final reaction mixture in a volume of 0.1 ml at 37°C contained 30 mM Tris-HCl, pH 7.45, 3 mM $MgCl_2$, 3 mM ATP, 1.0 μM [^{3}H]ouabain, and 50 μg of enzyme protein. The Na^+ concentration was varied as shown and ^2H_2O when present was at 85%. The reaction was initiated by the addition of [^{3}H]ouabain plus ATP.

Time of reaction	Na^+ (mM)	[^{3}H]Ouabain bound (mean±S.E.) (pmol/mg of protein)		k_{obs} (s^{-1})($\times 10^2$)		Inhibition (%)
		H_2O	^2H_2O	H_2O	^2H_2O	
20 s [a]	0	17.59±0.43	12.57±0.26	0.93	0.69	27
	8	31.58±0.37	25.10±0.72	1.57	1.23	22
	100	73.17±1.12	71.50±1.32	4.87	4.67	4
30 min [b]	0	100.73±0.01	98.16±0.18			3
	8	116.94±0.82	115.45±1.83			1
	100	117.61±1.73	117.76±1.60			0

[a] For inhibition at 20 s the values shown are the percent reductions in the observed pseudo-first order rate constants, calculated as described under Fig. 1.

[b] The percent inhibition at 30 min is the reduction in steady-state level.

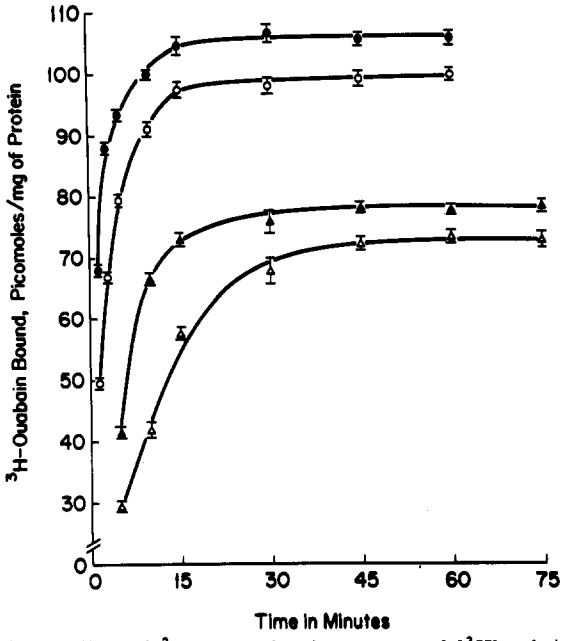

Fig. 1. Effect of ^2H_2O on the time course of [^{3}H]ouabain binding in the presence of $Mg^{2+} + P_i$. The basic reaction mixture contained 30 mM Tris-HCl, pH 7.45, 1 μM [^{3}H]ouabain ($1.5 \cdot 10^7$ dpm/μmol) and 50 μg of enzyme in a final volume of 0.1 ml. Other additions to various reactions were as follows: 5 mM $MgCl_2$ and 0.5 mM Tris-phosphate (○, H_2O; ●, 85% ^2H_2O); 20 mM $MgCl_2$ and 0.5 mM Tris-phosphate (△, H_2O; ▲, 85% ^2H_2O). The reaction, at 37°C, was initiated with [^{3}H]ouabain. At the indicated times, it was quenched with 3 ml of chilled 5 mM ouabain containing 30 mM Tris-HCl, pH 7.45 at 37°C. Nonspecific [^{3}H]ouabain binding for each reaction condition was measured by including 5 mM unlabeled ouabain present in the reaction, and was no greater than 2% of the steady-state level. The quenched samples were washed by Millipore filtration and radioactivity was determined by liquid scintillation spectrometry. Pseudo-first order rate constants for association phase of the reaction were calculated by using the equation

$$k_{obs} = -(1/t)\ln(((E\cdot O)_e - (E\cdot O)_t)/(E\cdot O)_e)$$

where $(E\cdot O)_e$ is the equilibrium value for enzyme-ouabain complex, and $(E\cdot O)_t$ is the amount of complex measured at a time during the linear phase of the reaction. The association constant k_1 was assumed to be much greater than dissociation constant k_{-1}, under the experimental conditions [25]. The rate constants (s^{-1}) obtained under various conditions were as follows: Mg^{2+} (5 mM)+P_i system: 0.0077 (H_2O solvent) and 0.0114 (^2H_2O solvent); Mg^{2+} (20 mM)+P_i system: 0.00159 (H_2O solvent), 0.00305 (^2H_2O solvent).

with these complexes. Since we had previously observed [7–10] that ^2H_2O inhibited reactions promoted by one conformation and stimulated those promoted by another, we decided to investigate the effects of ^2H_2O on types I and II binding, with the view that this could help elucidate conformations and pathways which might be involved in types I and II binding. As shown in Fig. 1, ^2H_2O was found to significantly stimulate [^{3}H]ouabain binding in the presence of $Mg^{2+} + P_i$. The pseudo-first order rate constant was enhanced by 48%, whereas the steady-state binding (at 30 min) was increased by 11%. The latter represents a small elevation in the binding capacity of the enzyme, presumably due to inhibition of dissociation and/or availability of additional binding sites. The effect of ^2H_2O was even more marked when the binding reaction was carried out in the presence of higher Mg^{2+} concentration. As shown in Fig. 2, ouabain binding in the presence of Mn^{2+} was also stimulated by ^2H_2O (60% of the pseudo-first order rate constant and 17% at steady-state). In contrast, ^2H_2O decreased the pseudo-first order rate constant for type I binding. There was no significant change in the steady-state level of binding under these conditions (Table I). Further, the inhibition was reduced as Na^+ was elevated in the reaction. This is in accord with the previously

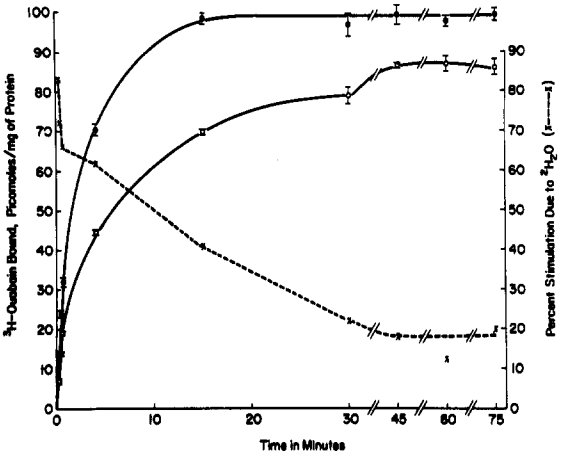

Fig. 2. Effect of ^2H_2O on the time course of [^{3}H]ouabain binding in the presence of Mn^{2+}. The experimental conditions were the same as those described in Fig. 1, except that 5 mM $MnCl_2$ was substituted for $MgCl_2 + P_i$, and the time of reaction was varied as shown. ○, H_2O system; ●, 85% ^2H_2O. The dashed line represents percent stimulation due to ^2H_2O over the time course of ouabain binding. Pseudo-first order rate constants (s^{-1}) in the Mn^{2+} system were: 0.00866 (H_2O solvent) and 0.01387 (^2H_2O solvent).

described effects of ^2H_2O on Na^+-dependent phosphoenzyme formation, where it was shown that the inhibition by ^2H_2O was diminished as the Na^+ concentration was increased [8,9].

Effect of pH and solvent on [^{3}H]ouabain binding

We investigated the binding reaction at different pH (or corresponding equivalent p^2H) in the medium, as it was essential to test the effects of pH shifts in relation to expected solvent effects. It is shown in Table II that varying the pH from 7.05 to 7.86, or the equivalent in p^2H from 7.39 to 8.20 [28], did not markedly affect the $Mg^{2+} + Na^+ + ATP$-dependent ouabain binding. On the other hand, there was a significant decrease in the type II ouabain-binding to the enzyme under similar conditions of altering pH or p^2H. Increasing pH from 7.05 to 7.86 and p^2H from 7.39 to 8.20 decreased ouabain binding (in the presence of $Mg^{2+} + P_i$) 75% and 58%, respectively (Table II). In contrast, as shown in Fig. 1, ^2H_2O actually stimulated the rate of ouabain binding by 48%. Therefore, significant stimulation persisted, despite possible inhibitory effects exerted by elevated pH due to ^2H_2O. In the case of Mn^{2+}-dependent binding, each 0.4 unit increment in pH (from 7.05 to 7.86) increased ouabain binding by 20–24%. Substitution of H_2O by ^2H_2O increased the alkalinity less than 0.4 pH unit (from pH 7.45 to p^2H 7.79), but, as shown in Fig. 2, resulted in an 83% stimulation of binding. Hence, at least 59% of this stimulation cannot be attributed to a solvent-induced increase in alkalinity. Although changes in pH had opposite effects on $Mg^{2+} + P_i$-, and Mn^{2+}-dependent ouabain binding, the final effect of ^2H_2O was the same for both, i.e., stimulatory. The above effect, therefore, represents a real stimulatory (or inhibitory) effect of the solvent on ouabain binding to the enzyme. Further, under comparable experimental conditions (especially in the presence of H_2O) our results on the relation of pH and [^{3}H]ouabain binding are in accord with those reported by others [26,27].

Effect of the order of addition of Me_2SO and ligands on ouabain binding

In previous work we demonstrated that effects of Me_2SO on reactions of the phosphoenzyme intermediate were analogous to those of ^2H_2O [8–10]. Accordingly, we examined the actions of Me_2SO on various types of ouabain binding to the enzyme. It is shown in Table III (Expt. 1) that Me_2SO potently inhibited (i.e., even more so than ^2H_2O) type I ouabain binding. This inhibition was considerably enhanced when Me_2SO was allowed contact with the enzyme prior to the addition of other ligands. On the other hand, Me_2SO stimulated (to an even greater extent than ^2H_2O) the type II binding of ouabain to the enzyme (Table III, Expt. 2). Prior contact of the enzyme with the ligands resulted in a relatively greater stimulation than when Me_2SO was allowed to act on the enzyme first, and may be a result of a direct inhibitory effect of Me_2SO on ouabain- or metal-

TABLE II

EFFECT OF pH AND p^2H ON BINDING OF [^{3}H]OUABAIN IN THE PRESENCE OF VARIOUS LIGANDS

The experimental conditions were the same as described in the legends to Table I and Fig. 2, except that the pH or p^2H were varied as shown. Where present, Na^+ concentration was 8 mM. The reaction time for the $Mg^{2+} + Na^+ + ATP$ system, and for Mn^{2+} system was 20 s, whereas for the $Mg^{2+} + P_i$ system it was 1.5 min.

pH	[^{3}H]Ouabain bound (pmol/mg of protein) (H_2O as solvent) in the presence of			p^2H	[^{3}H]Ouabain bound (pmol/mg of protein) (^2H_2O as solvent) in the presence of	
	$Mg^{2+} + Na^+ + ATP$	$Mg^{2+} + P_i$	Mn^{2+}		$Mg^{2+} + Na^+ + ATP$	$Mg^{2+} + P_i$
7.05	34.38 ± 0.99	78.04 ± 2.10	11.2 ± 0.30	7.39	24.68 ± 1.26	76.60 ± 1.76
7.45	32.10 ± 0.31	47.74 ± 1.55	13.97 ± 1.25	7.79	24.34 ± 0.36	56.10 ± 1.00
7.86	29.07 ± 0.67	19.74 ± 0.15	17.35 ± 0.57	8.20	25.49 ± 0.51	32.48 ± 0.38

TABLE III

EFFECT OF ORDER OF ADDITION OF Me_2SO AND LIGANDS ON [^{3}H]OUABAIN BINDING

Experiment 1: 50 μg of enzyme protein was mixed with Tris-HCl, pH 7.45 at 37°C and $MgCl_2$. Other additions were then made in the order indicated. In a final volume of 0.1 ml, the concentrations of various components of the reaction medium were Tris-HCl (30 mM), $MgCl_2$ (3 mM), ATP (3 mM), Na^+ (100 mM), Me_2SO (30%) and [^{3}H]ouabain (1 μM). Control ouabain binding value with H_2O as solvent was 91.02±1.99 (mean±S.E.) pmol/mg protein. Nonspecific binding 1.52±0.11 pmol/mg protein, was unaffected by the various treatments. Time of contact with various ligands was as shown; s, seconds; [^{3}H]O, [^{3}H]ouabain. Experiment 2: The reaction conditions were the same as in Experiment 1, except that final concentration of various components of the reaction medium were: $MgCl_2$ (5 mM), Tris-P_i (0.5 mM), and $MnCl_2$ (5 mM). Control [^{3}H]ouabain binding with Mn^{2+} was 8.75±0.41 and with Mg^{2+} + P_i was 5.97±0.07 pmol/mg of protein. Control binding with Mg^{2+} alone was marginally above background; therefore, stimulation was calculated as in Experiment 1. Each experiment was independently controlled.

Sequence of addition	Percent inhibition (−) or stimulation (+)
Experiment 1	
E+ATP (10 s)+(Me_2SO, Na^+, [^{3}H]O) (20 s)	−59
E+Na^+ (10 s)+(Me_2SO, ATP, [^{3}H]O) (20 s)	−61
E+(ATP, Na^+) (10 s)+(Me_2SO, [^{3}H]O) (20 s)	−61
E+(Me_2SO, ATP, Na^+, [^{3}H]O) (20 s)	−60
E+Me_2SO (10 s)+(ATP, Na^+, [^{3}H]O) (20 s)	−89
Experiment 2	
E+Me_2SO (10 s)+(Mg^{2+}, P_i, [^{3}H]O) (20 s)	+318
E+Mg^{2+} (10 s)+(Me_2SO, P_i, [^{3}H]O) (20 s)	+409
E+P_i (10 s)+(Me_2SO, Mg^{2+}, [^{3}H]O) (20 s)	+422
E+(Mg^{2+}, P_i) (10 s)+(Me_2SO, [^{3}H]O) (20 s)	+405
E+Me_2SO (10 s)+(Mg^{2+}, [^{3}H]O) (20 s)	+370
E+Mg^{2+} (10 s)+(Me_2SO, [^{3}H]O) (20 s)	+740
E+Me_2SO (10 s)+(Mn^{2+}, [^{3}H]O) (20 s)	+27
E+Mn^{2+} (10 s)+(Me_2SO, [^{3}H]O) (20 s)	+98

binding sites of the enzyme (as e.g., in the type I binding). However, once type II conformation was promoted, it was further augmented by Me_2SO.

Discussion

The results described herein demonstrate that the rates of ouabain binding to the (Na^+,K^+)-ATPase are either stimulated or inhibited by ^2H_2O and Me_2SO depending upon the nature of ligands present. It is well known that the conformation of the enzyme may influence complex formation with ouabain [17,22,23,29], and that the rate of association of ouabain, more than the rate of dissociation, is affected by ligands [25]. Agents, such as ^2H_2O and Me_2SO, may also exert their effects on enzyme-ligand-ouabain complexes through modulation of the enzyme conformation and hence the rates of association or dissociation.

Under type I binding conditions (Mg^{2+} + ATP + Na^+), the enzyme undergoes several reactions and interconversions resulting in phosphorylated intermediates E_1-P and E_2-P, while possibly containing nonphosphorylated forms E_1 and E_2 as well [17,30]. Although the existence of E_1 and E_2 under these conditions may not have been specifically demonstrated, it is implied that if E_1-P and E_2-P undergo spontaneous dephosphorylation (in the absence of K^+), the immediate products are E_1 and E_2, respectively [17,18,21]. Also, it is understood that E_1 is a prerequisite for E_1-P and is promoted by ATP, Na^+ or ATP + Na^+ prior to phosphorylation [9].

In the presence of ligands supporting type II ouabain complexes, E_2 and E_2-P probably exist [18,31]. However, with Mg^{2+} or Mn^{2+} without added P_i, the enzyme should not exist in a phosphorylated state except for possible low levels of contaminating P_i. In the case of Mn^+, the amount of steady-state binding was comparable to that

obtained with $Mg^{2+} + P_i$, therefore it does not seem feasible that a significant fraction of the Mn^{2+}-E-ouabain complex could contain P_i. It might be argued that some P_i could be generated during the binding reaction with $Mg^{2+} + ATP + Na^+$, producing the type II binding. However, it is not likely that a significant amount of P_i would be formed in 20 s, the period in which our rate studies were performed. Also, type I binding was inhibited by ^2H_2O, whereas type II (with P_i) was stimulated, therefore at least a portion of type I must not be due to P_i. In the absence of exogenous Na^+, with only $Mg^{2+} + ATP$ (Tris salt) we observed also a significant binding in 20 s with maximal inhibition by ^2H_2O. It is unlikely that under this condition an appreciable amount of P_i (or E_2-P) was formed. Therefore, it appears that type I complexes involve at least some E_1 forms of the enzyme. Thus we conclude, in agreement with others, that under appropriate ligand conditions ouabain may associate with multiple forms of the enzyme, including nonphosphorylated forms E_1, E_2 [17,27,32] and phosphorylated forms (E_1-P, E_2-P) [17,22].

It has been proposed that E_2-P was the major form to which ouabain association occurred. However, the enzyme system employed for this work may have predominated in E_2-P as its phosphorylated state [17]. In contrast, other sources of enzyme have been shown to have a higher fraction of E_1-P out of the total phosphoenzyme produced from ATP [29], as is the case with the system reported here [10]. Evidence for ouabain complex with E_1-P may not have been observed by Tobin et al. [29], because phosphorylation and binding conditions were not precisely the same, and because there may be a difference in the efficacy of Ca^{2+} and Mg^{2+} to support ouabain binding, as indicated by the fact that when Ca^{2+} was substituted equally for Mg^{2+} in the $Mg^{2+} + P_i$-dependent pathway, only 8% as much ouabain was bound (see their Table III). The corresponding efficacy of Ca^{2+} and Mg^{2+} to produce E_2-P from P_i apparently was not studied. Therefore, there is no definitive proof that ouabain cannot also associate with E_1-P.

Based on the above considerations, we propose the scheme shown in Fig. 3. Solvents such as ^2H_2O and Me_2SO having hydrophilic activities appear to promote a shift to the right in the equilibrium $E_1 \rightleftarrows E_2$ whether the enzyme is phosphorylated or not [8–10]. As illustrated in Fig. 3, this action would stimulate activities on which E_2 or E_2-P are dependent and inhibit those on which E_1 and E_1-P are dependent. Furthermore, as shown previously, Na^+ promotes E_1 form of the enzyme in competition with the hydrophilic actions of solvents [9,33]. As was observed, Na^+-dependent activities of the enzyme, including ouabain binding, should be inhibited by ^2H_2O and Me_2SO in an apparently competitive manner with respect to Na^+. Therefore, Na^+-dependent binding of ouabain may involve E_1 forms of the enzyme (E_1 and E_1-P), consistent with the observations of Siegel and Fogt [32]. The arguments for ouabain complexes with

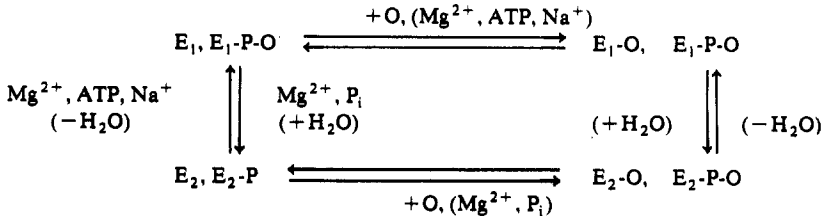

Fig. 3. Proposed scheme for ouabain (O) binding and the role of H_2O in (Na^+,K^+)-ATPase. E_1, E_1-P are promoted by Na^+; E_2, E_2-P are promoted by Mg^{2+}, P_i (or Mg^{2+} or Mn^{2+}). These conformations are defined as in Ref. 3. More than one conformational species of the enzyme may be present in each step, and may arise under either of the binding type conditions as indicated by the reversible and cyclical nature of the scheme. E_1-O and E_1-P-O represent pathways of type I binding, inhibited by ^2H_2O and Me_2SO, i.e. are promoted by removal of H_2O. E_2-O and E_2-P-O represent pathways of type II binding complexes, stimulated by ^2H_2O and Me_2SO, i.e. are enhanced by hydrophilic conditions. An interconversion of E_1-O and E_2-O is postulated; if this could be tested directly, it would be interesting to see if the interconversions of E_1-O and E_2-O would similarly be promoted by the absence or presence of bound H_2O, respectively. It is not intended to imply that the only difference between enzyme conformations 1 and 2 is due to bound H_2O. Rather, the association-dissociation of H_2O may play a role in assisting or inhibiting the configurational changes induced by various ligands.

E_2 and E_2-P have been given above and the scheme illustrates how ^2H_2O and Me_2SO may stimulate those activities, in agreement with previously observed actions of these solvents [8–10].

The effects of order of addition of Me_2SO and ligands on ouabain binding also agree with the reported effects on reactions involving the phosphorylated intermediate [9,10]. The degree of inhibition of type I binding and E-P formation was roughly equivalent, either when Me_2SO contacted the enzyme first, or when Me_2SO and ligands contacted the enzyme simultaneously (see, for example, Table III in this article, and Table II in Ref. 9). For both reactions, maximal inhibition was obtained when Me_2SO contacted the enzyme first. One difference was that when ATP, Na^+, or both contacted the enzyme before Me_2SO there was no inhibition of E-P formation, whereas this sequence of additions inhibited type I binding to the same degree (60%) as did the simultaneous addition. Thus, Me_2SO may inhibit type I binding when it would not inhibit phosphorylation from ATP. This indicates that solvent may inhibit ouabain association with its receptor site, a point which was further indicated by the order of addition effect on type II binding. In the latter case, Me_2SO stimulated the least when it contacted the enzyme before the supporting ligands. In regard to stimulation of type II binding, this agrees with effects of Me_2SO and ^2H_2O on K^+-dependent activities involving the phosphoenzyme or substrate hydrolysis [10]. When these solvents were added to E-P after it was formed, they enhanced the reaction of K^+ with the K^+-sensitive component of E-P (E_2-P).

In summary, based on the above described observations and previous results [7–10], we have proposed a mechanism correlating the multiple effects of these solvents and a role of water in regulating the activities of this enzyme (Fig. 3). It is noteworthy that a role of H_2O in the conversion of 'low energy' and 'high energy' phosphoenzyme of sarcoplasmic reticulum Ca^{2+}-ATPase has also been proposed recently [34].

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