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The effect of dimethylsulfoxide on the substrate site of Na^+/K^+-ATPase studied through phosphorylation by inorganic phosphate and ouabain binding

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Abstract

To obtain further information on the role of H_2O at the substrate site of Na^+/K^+-ATPase, we have studied the enzymes reaction with P_i and ouabain in 40% (v/v) Me_2SO (dimethylsulfoxide). When the enzyme (E) was incubated with ouabain (O) for 5 min in a 40% (v/v) Me_2SO-medium with 5 mM $MgCl_2$ and 0.5 mM KCl (but no phosphate), ouabain was bound (as EO). Subsequent incubation with P_i showed that E, but not EO, was rapidly phosphorylated (to EP). Long-time phosphorylation revealed that EO is also phosphorylated by P_i albeit very slowly ($t_{1/2}$ about 60 min) and that binding of ouabain to EP also is very slow. The EOP complex is stable, i.e., the $t_{1/2}$ for the loss of P_i is $\gg$ 60 min in contrast to about 1 min in water. These results in 40% Me_2SO are distinctly different from what would be obtained in a watery milieu: ouabain would bind slowly and inefficiently in the absence of P_i, and ouabain would catalyse phosphorylation from P_i rather than retard it. Equilibrium binding of [^{3}H]ouabain to E and EP in water or 40% Me_2SO confirmed these observations: K_{diss} in water is 11 μM and 12 nM for EO and EOP, respectively, whereas in Me_2SO they are 112 nM and 48 nM. It is suggested that the primary effect of the lowered water activity in 40% Me_2SO is a rearrangement of the substrate site so that it also in the absence of P_i attains a transition state configuration corresponding to the phosphorylated conformation. This would be sensed by the ouabain binding site and lead to high affinity ouabain binding in the absence of P_i.

Keywords: ATPase, Na^+/K^+-; Dimethylsulfoxide; Phosphoenzyme from P_i; Ouabain binding

1. Introduction

All the known cation transport ATPases, including Na^+/K^+-ATPase (EC 3.6.1.37) with which we are concerned in this paper, operate with a mechanism where the hydrolysis of ATP proceeds via formation of an acid anhydride between phosphate and an aspartyl residue at the enzymes substrate site [1] (the term 'substrate site' is used here for the domains involved in the binding of ATP and in the subsequent phosphorylation). The resulting phosphorylated enzyme intermediates (EP) play a crucial role in the common reaction scheme for Na^+/K^+-ATPase [2] where their formation, catalysed by Mg^{2+} and Na^+, and breakdown, catalysed by K^+, are important steps in the energy transduction and cation transport. Interestingly enough, the enzyme can also form a phosphorylated intermediate (at the same aspartyl residue) by reaction with Mg^{2+} and inorganic phosphate, P_i [2].

We have previously described [3,4] how the phosphorylation of Na^+/K^+-ATPase from ATP changes properties when the activity of water is lowered by addition of 40% Me_2SO to the medium: in the absence of Me_2SO, this phosphorylation is catalysed by Na^+ and counteracted by K^+ as mentioned above, but with 40% Me_2SO in the medium, phosphorylation from ATP takes place in the absence of Na^+ and is actually activated by 0.1–0.5 mM K^+. In the evaluation of these studies it was suggested that Me_2SO (through lowered water activity) affects both the substrate site of Na^+/K^+-ATPase and the cation binding sites.

The purpose of the present work is to obtain further information on the role of water for the reactions of

Abbreviations: Me_2SO, dimethylsulfoxide; EP, phosphoenzyme; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; P_i, inorganic orthophosphate.

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Na^+/K^+-ATPase, notably for the properties of the substrate site and for the extracellular aspects of the pump. To achieve this, we have studied the pump primarily under conditions where it is not turning over, i.e., we have investigated the relationship between phosphorylation from P_i at the substrate site and ouabain interaction with the extracellular aspects of Na^+/K^+-ATPase. In aqueous media, ouabain reacts preferentially with one (or more) phosphoenzyme(s) obtained by phosphorylation in ATP + Na^+ + Mg^{2+}-media or in P_i + Mg^{2+}-media [2,5,6]. In this study we show, that with the lowered water activity obtained by addition of 40% Me_2SO, this picture is altered, and that especially the phosphorylation site of the enzyme is affected by the decrease in water activity.

2. Experimental procedures

2.1. Enzyme preparations

Purified Na^+/K^+-ATPase was prepared from pig kidney outer medulla using the procedures of Jørgensen [7] with the modifications described by Jensen et al. [8]. The ATPase activity for the different preparations (V_{max}, [9]) was between 10 and 20 units mg^{-1}. From the measured phosphorylation capacity and the ouabain binding capacities reported in this paper (e.g., Fig. 8) and elsewhere [10], the turnover number of these enzyme preparations were 9000–10000 min^{-1}, indicative of an undenatured, functional enzyme [7,10]. For further details, see [3] and [4]. Protein was determined by the method of Lowry et al. [11].

2.2. Phosphorylation experiments

All experiments were carried out at room temperature (27° C). In the phosphorylation experiments with radioactive, inorganic phosphate ($^{32}P_i$) or with [γ-^{32}P]ATP, with or without ouabain, the standard Me_2SO-medium contained 40% Me_2SO (v/v), 5 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$ and 0.5 mM KCl. Other conditions, like time and procedures for preincubations and additions, appear in the figure legends. The amount of phosphoenzyme, EP, was determined after acid precipitation as described [3].

2.3. Ouabain binding

Bound ouabain was determined by filtration on Millipore filters, pore size 0.45 μm, which were then washed four times with portions of 4 ml of a chilled standard Me_2SO-medium without ouabain. The concentration of unbound ouabain was calculated as the difference between total (added) and bound ouabain. The radioactivity (^3H) associated specifically with ouabain in the [^{3}H]ouabain stock solution was determined by measuring unbound radioactivity in the supernatant after centrifugation of a diluted sample of [^{3}H]ouabain incubated with a large excess of Na^+/K^+-ATPase [12,13]. The 'unspecific' radioactivity was 7–9% of the total. Blanks for specific ouabain binding were obtained by a 5 min preincubation of enzyme with 1 mM non-radioactive ouabain before the addition of [^{3}H]ouabain. The blanks were less than 2% of maximal binding.

2.4. Chemicals

[γ-^{32}P]ATP was prepared by the method of Glynn and Chapell [14] as described [3]. $^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and purified according to Kessler et al. [15]. Ouabain was from Sigma and [^{3}H]ouabain was purchased from NEN and used without further purification (but see above for determination of 'unspecific radioactivity'). Tris and other buffers, KCl, $MgCl_2$, and EDTA (all analytical grade) were from Sigma, and Me_2SO (spectroscopic grade) was purchased from Merck.

3. Results

3.1. Preliminary experiments on phosphorylation from P_i

Since this study with Me_2SO is concerned with the interaction of both the dephosphoenzyme and EP with ouabain, it was necessary briefly to study conditions for phosphorylation of Na^+/K^+-ATPase by P_i in 40% Me_2SO. It has been shown [16,17] that in 25–40% Me_2SO, the apparent affinity for P_i is increased by a factor of up to 200 relative to that in water, so that the apparent dissociation constant is in the μM range. Furthermore, the phosphorylation reaction requires millimolar concentrations of Mg^{2+} [18]. In connection with our earlier studies on the effect of Me_2SO on phosphorylation from ATP (which requires no Na^+, and is stimulated by low concentrations of K^+ [3,4]), we found that phosphorylation from P_i was also stimulated by 0.5 mM KCl, Fig. 1 in [4]. In the present study we could confirm the K^+-effect: in 40% Me_2SO with 5 mM MgCl, the addition of 0.5 mM KCl resulted in a considerably increased rate of phosphorylation from phosphate, $t_{1/2}$ decreasing from more than 100 s with no K^+, to less than 10 s with 0.5 mM K^+, and the rate became even higher with 100 mM KCl (not shown). With saturating P_i (20 μM), the steady-state EP level increased with KCl up to 0.3 mM whereafter it remained constant. In contrast, in the absence of Me_2SO ('aqueous media'), the EP level is maximal without KCl, decreases sharply to about 50% in 0.5 mM KCl and is almost zero at 100 mM KCl (not shown), which is in accordance with earlier observations [5,12].

As a consequence of this, the reaction mixtures in the phosphorylation experiments (with 40% Me_2SO) in the present work contain 0.5 mM KCl and, to satisfy the Mg^{2+}-requirement, also 5 mM $MgCl_2$. Since the relation-

ship of ouabain binding and phosphorylation was investigated in this paper, the experiments with ouabain also contain 0.5 mM KCl and 5 mM $MgCl_2$.

3.2. Preincubation with ouabain in the absence of P_i and subsequent phosphorylation by P_i (short-time-experiments)

When Na^+/K^+-ATPase (E) is incubated in an aqueous milieu with ouabain (O) in the absence of phosphorylating ligands (ATP + Na^+ + Mg^{2+} or P_i + Mg^{2+}), the EO complex is formed very slowly and in a low yield [12,19,20]. In the presence of Mg^{2+} alone, some binding is usually observed [12,19–21] but if the enzyme becomes phosphorylated, e.g., by the simultaneous action of P_i + Mg^{2+} (or ATP + Na^+ + Mg^{2+}), optimal conditions for ouabain binding are obtained [6,12,19–21] (see also [5] for further references). With 40 vol% Me_2SO in the medium, the pattern is quite different as illustrated in the following.

In the experiments shown in Fig. 1, the enzyme was preincubated with 5 mM MgCl without (open circles) or with 5 μM ouabain (closed circles), and then 20 μM P_i was added to initiate phosphorylation at time 0 on Fig. 1. Firstly, there is a rapid phosphorylation during the first 10 s, and generally, the time course seems unaffected by ouabain, suggesting that it is the same enzyme species that is being phosphorylated in the two cases. Only about 35% of the ouabain incubated enzyme seems to be available for fast phosphorylation, and this possibly indicates that about 65% of the enzyme has reacted with ouabain to form EO during the 5 min preincubation. Secondly, there is only a very slow increase in EP after 15 s both in the presence and in the absence of ouabain in this short-time experiment and the % inhibition of EP-formation seems to be independent on time (Fig. 1, inset). This suggests that the EO formed during preincubation is not (or only very slowly)

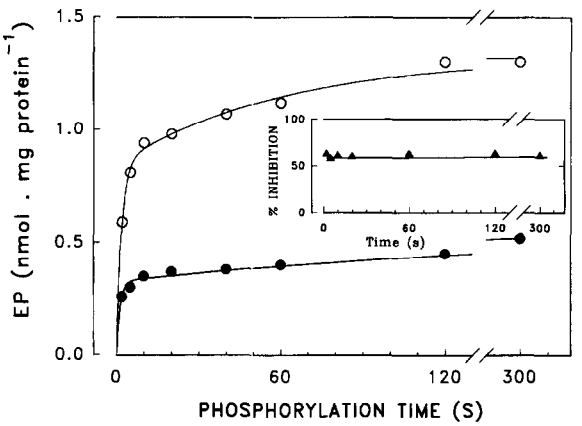

Fig. 1. Time course of the phosphorylation reaction in the presence and absence of ouabain. Na^+/K^+-ATPase (0.1 mg ml^{-1}) was preincubated for 5 min in the standard Me_2SO-medium (see Experimental procedures) in the absence (○) or in the presence of 5 μM ouabain (●). Phosphorylation was initiated by addition of 20 μM $^{32}P_i$ and EP was precipitated with acid (Experimental procedures) at the times indicated. The inset shows the percentage inhibition of EP-formation by ouabain.

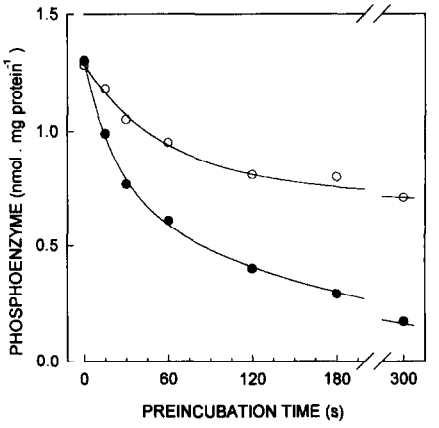

Fig. 2. The time of preincubation with ouabain and the resulting inhibition of the rapid phosphorylation by P_i. Na^+/K^+-ATPase (0.09 mg ml^{-1}) was preincubated for 0–5 min in the standard Me_2SO-medium with 5 μM (○) or 15 μM (●) ouabain. At the times indicated, 20 μM $^{32}P_i$ was added and 1 min hereafter EP was precipitated by acid and measured.

phosphorylated by P_i during the 300 s of the experiment (but see long-time experiments below).

Similar conclusions were reached from experiments (not shown) where the enzyme was preincubated for 5 min with or without 5 μM ouabain and subsequently phosphorylated for 1 min by P_i in concentrations from 0 to 100 μM. The $K_{0.5}$ for P_i was about 2 μM independently of the presence or absence of ouabain, but the EP-level was decreased by a factor of 3 by preincubation with 5 μM ouabain. Both these experiments show that ouabain binds to the enzyme in the absence of added P_i, and that only free (non-ouabain-bound) enzyme reacts with P_i during the subsequent 1–2 min phosphorylation period.

The degree of ouabain-blockage of phosphorylation, which thus probably signifies the degree of ouabain-binding to an EMgK complex during preincubation, is both dependent on the time of preincubation with ouabain and on the ouabain concentration (Figs. 2 and 3B). Like in water [5,6], the effect of ouabain, i.e., ouabain binding, develops slowly (of course depending on [ouabain]) and equilibrium binding is approached, but hardly achieved, after 5 min (Fig. 2). This means that the $K_{0.5}$ of 5 μM, which one might be tempted to deduce from Fig. 3B, is most certainly different from the ouabain-enzyme dissociation constant (see later for results of ouabain binding experiments).

To emphasise the difference between the reactions with ouabain and with P_i in water and in Me_2SO, we preincubated the enzyme for 5 min with or without ouabain, in water or 40% Me_2SO, and we then followed the subsequent formation of EP from P_i. Fig. 3 clearly illustrates two aspects of the role of Me_2SO for the interaction between the enzyme, inorganic phosphate and ouabain: the first point is, that in the absence of ouabain, the phosphorylation by P_i in μmolar concentrations is much lower in water than in Me_2SO (where close to the maximal EP

level for this enzyme preparation is attained). This is most likely due to the fact that the affinity for P_i is much lower in water than in 40% Me_2SO [16]. Secondly, whereas incubation with ouabain (before and during the phosphorylation period) in a watery milieu stimulates the phosphorylation with P_i (Fig. 3A, and [22–25]), preincubation with ouabain in Me_2SO clearly inhibits the following phosphorylation from P_i (Fig. 3B, see also Figs. 1 and 2). This means that the enzyme-ouabain complex (EO) is phosphorylated very slowly, if at all, so that in contrast to the situation in water, very little EOP is formed in 40% Me_2SO in these short-time experiments.

At this point one might speculate if contamination with inorganic phosphate, P_i, could play a role for the results obtained so far, especially considering the fact that Na^+/K^+-ATPase has a high affinity for P_i in the presence of 5 mM $MgCl_2$ and 40% Me_2SO. We have investigated this point using different approaches. First of all we tried to measure [P_i] in the reagents and enzyme preparations using the very sensitive (down to 0.25 nmol, corresponding to about 0.5 μM in the samples) malachite-green method [26]. It was not possible to measure directly P_i in Me_2SO, because of interference with the colour development, but a 40% Me_2SO-medium with enzyme, Tris, $MgCl_2$ and KCl gave values corresponding to the lower limits of detection. In a second approach we phosphorylated the enzyme in subsaturating, added concentrations of P_i, like 1 or 3 μM. Judged from the yield of $E^{32}P$ and the calculated specific activities we also here obtained 0.5 μM as the upper limit of contamination. Finally, we measured the effect of 'contaminating' the preincubation medium with known concentrations of P_i, on the inhibition of the subsequent phosphorylation (in experiments like

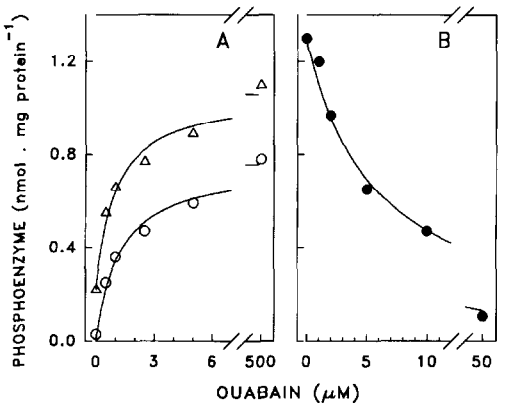

Fig. 3. Comparison between the interaction with ouabain and phosphorylation from P_i in a watery milieu (A) and in 40% Me_2SO (B). Na^+/K^+-ATPase (0.07 mg ml^{-1}) was preincubated for 5 min in a medium with 5 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 0.5 mM KCl and the ouabain concentrations shown on the abscissa (0–500 μM in water, panel A and open symbols; 0–50 μM in Me_2SO, panel B and closed symbols). The phosphorylation was initiated by addition of $^{32}P_i$ in concentrations of 5 μM (○), 50 μM (△) or 20 μM (●), and after 2 min, EP was precipitated by acid and determined as described under Experimental procedures.

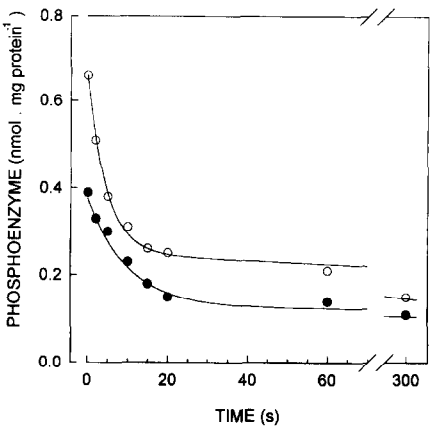

Fig. 4. Effect of preincubation with ouabain in 40% Me_2SO on the dephosphorylation of EP formed from P_i during 1 min. Na^+/K^+-ATPase (0.3 mg ml^{-1}) was preincubated for 5 min in the Me_2SO-medium with 10 μM ouabain (●) or without ouabain (○) and then phosphorylated for 1 min with 20 μM $^{32}P_i$. Dephosphorylation of $E^{32}P$ was initiated at time = 0 by a 20-fold dilution with the same media but with non-radioactive P_i, and at the times indicated, samples were precipitated with acid and $E^{32}P$ determined as described. The dephosphorylation rate coefficient was about 0.1 s^{-1} in both experiments.

Fig. 1). In a 10 min incubation with 30 μM ouabain we noticed only a marginal increase in apparent ouabain binding by adding 3 μM P_i. We infer from these experiments that any P_i-contamination is so small that it has no influence on the conclusions drawn from the present study.

The hypothesis based on the above relatively short-time experiments is, that in Me_2SO, and irrespective of the preincubation with ouabain or not, only one form of phosphoenzyme (EP and not EOP) is formed after addition of P_i. This is corroborated by the dephosphorylation experiment shown in Fig. 4: After 5 min preincubation and subsequently 1 min phosphorylation with $^{32}P_i$ (time 0 in Fig. 4), the EP level is lowest in the samples incubated with ouabain, but the dephosphorylation rate coefficient is ouabain independent.

3.3. The effect of ouabain on long-time phosphorylation by P_i in 40% Me_2SO

To investigate whether the enzyme-ouabain complex, EO, could be phosphorylated by P_i and form EOP when the phosphorylation period was extended to longer times, the following experiments were made: Na^+/K^+-ATPase was preincubated in the 40% Me_2SO-medium for 5 min with or without ouabain, then $^{32}P_i$ was added and phosphorylation was allowed to proceed for up to 120 min. As seen from Fig. 5A and B, and in accordance with the previous results, there is a rapid phase of EP formation, the magnitude of which is dependent on the ouabain concentration. Hereafter there is a slow increase in $E^{32}P$ formation so that the ouabain incubated samples approach the EP level for the experiments without ouabain. It appears that EO is formed during preincubation with ouabain, E is rapidly

phosphorylated to EP, whereas EO reacts only slowly with P_i to form EOP. According to this model, the difference between the upper curve (no ouabain) and the lower curve (with ouabain) in Fig. 5 represents EO. Whether the rapidly formed EP is converted to EOP during the phosphorylation period is addressed below.

The dephosphorylation experiments shown in Fig. 6A and B support the idea that prolonged incubation in 40% Me_2SO of the enzyme with both ouabain and P_i results in formation of a phosphoenzyme which is different from EP. This phosphoenzyme is presumably EOP. It is formed slowly (Fig. 5) and it is much more stable than EP (Fig. 6A and B). After 20 min incubation with both ouabain and P_i the phosphoenzyme that would dephosphorylate rapidly has disappeared and there seems to be only one type of, slowly dephosphorylating, phosphoenzyme present. This must be EOP and this suggests that not only will EO react with P_i, but EP can also bind ouabain, albeit slowly. This conclusion is supported by the direct measurements of ouabain-binding described below.

3.4. Ouabain inhibition of phosphorylation from ATP

In two previous studies [3,4] we have shown that the enzyme is phosphorylated in Me_2SO by ATP in the presence of 5 mM $MgCl_2$ and 0.5 mM KCl (but in the absence

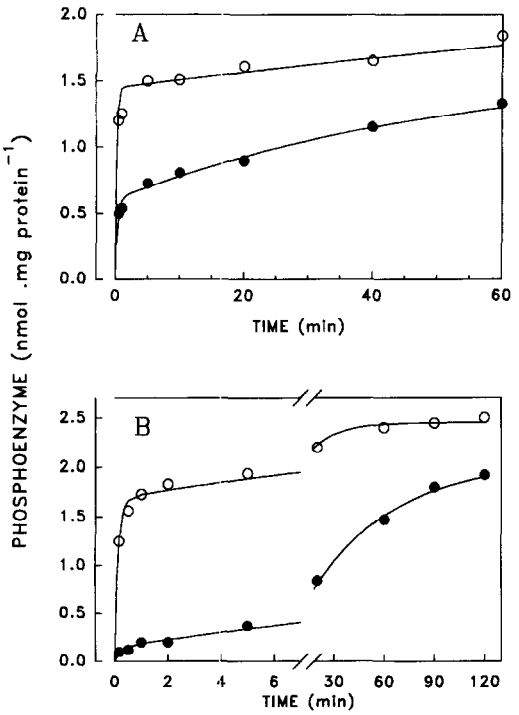

Fig. 5. Long-time phosphorylation by P_i in 40% Me_2SO of enzyme preincubated with or without ouabain. Na^+/K^+-ATPase (0.08 mg ml^{-1}) was preincubated for 5 min in the Me_2SO-medium without ouabain (○) or with 100 μM ouabain, panel A (●), or 1 mM ouabain, panel B (●). Then 20 μM $^{32}P_i$ was added and phosphorylation allowed to proceed as indicated.

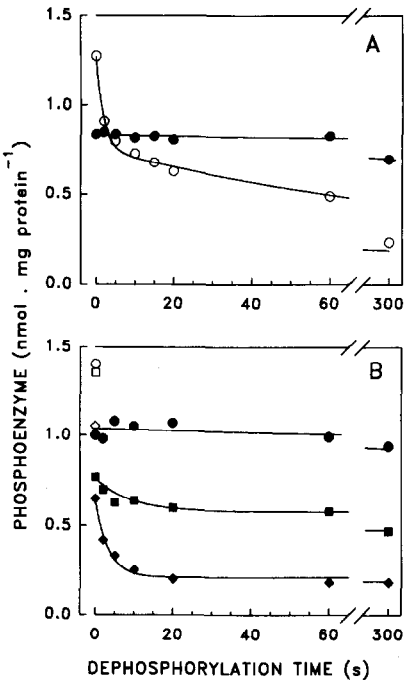

Fig. 6. Dephosphorylation characteristics of phosphoenzyme formed in 40% Me_2SO by 1 to 20 min incubation of Na^+/K^+-ATPase with P_i, with or without ouabain. Except for the varying incubation times with P_i, the experimental procedure was essentially as described in Fig. 4. (A) The enzyme was preincubated for 5 min without (○) or with 10 μM ouabain (●), then phosphorylated for 20 min with 50 μM $^{32}P_i$ whereafter the sample was diluted 20-fold with the same medium (with non-radioactive P_i). Samples were analyzed for remaining $E^{32}P$ at the times indicated. (B) As in panel A, only the phosphorylation period was 1 min (◆), 5 min (■) or 20 min (●). Open symbols represent EP levels after 1 (◇), 5 (□) or 20 min (○) phosphorylation in the absence of ouabain.

of Na^+), i.e., reaction conditions similar to those used in the present study. Fig. 7 shows that preincubation with ouabain prevents this phosphorylation from ATP in much the same way as ouabain prevents phosphorylation from P_i. The $K_{0.5}$ for ouabain (compare Fig. 3B and Fig. 7A) is the same for the two ouabain effects. Fig. 7B confirm our previous finding that the $K_{0.5}$ for ATP is a few μM, Fig. 5 in [3], and furthermore shows that this value is ouabain independent.

The role of Na^+ in the Me_2SO-medium was briefly investigated (results not shown). All preincubations with or without ouabain were of 5 min duration with 5 mM $MgCl_2$ but here no KCl was present. Without ouabain, 100 mM Na^+ in the preincubation leads to a more readily phosphorylated (from ^{32}P-ATP) enzyme species than if Na^+ is omitted. The lack of Na^+ in the preincubation is not (or at least only partly) made up for by its presence in the following 15 s of phosphorylation with ATP. As in Fig. 7A, preincubation with ouabain prevented phosphorylation, and it is particularly interesting for comparison with the reactions in water, that 100 mM Na^+ did only slightly, if at all, influence the binding of ouabain to the enzyme.

3.5. Direct measurements of ouabain binding to E and EP in 40% Me_2SO

The effect of ouabain in the phosphorylation studies with $^{32}P_i$ and [γ-^{32}P]ATP were naturally assumed to be due to ouabain binding to the enzyme, and the ouabain-binding studies reported in the following directly support this theory. The purpose of these ouabain-binding studies were twofold: to investigate the binding of ouabain to phosphorylated enzyme (EP) and to dephosphoenzyme (E) and to compare the characteristics of ouabain binding in water and in 40% Me_2SO. When the time course of ouabain binding to E and EP was studied in 40% Me_2SO (results not shown), we found that it was concentration dependent and quite similar for binding to E and EP and, as in a watery milieu [5,6], it took minutes to hours to reach binding equilibrium, the lower the ouabain concentration the longer the time. Consequently the samples were incubated with ouabain for 5–8 h before bound and free ouabain was measured.

The results of these equilibrium binding experiments are given in Fig. 8A (aqueous milieu) and 8B (40% Me_2SO). In water there is, in accordance with what is well known from the literature, a much higher affinity for ouabain to EP than to the dephosphoenzyme E (here 10^3-times higher, $K_d = 12$ nM versus 11 μM), i.e., phosphorylation promotes ouabain binding. In 40% Me_2SO the difference is much smaller since ouabain binds to EP with $K_d = 48$ nM and to E with $K_d = 112$ nM. In all the experiments reported here, B_{max} was close to 2 nmol mg^{-1} and the binding isotherms could be described by binding to a single population of sites (full curves in Fig. 8).

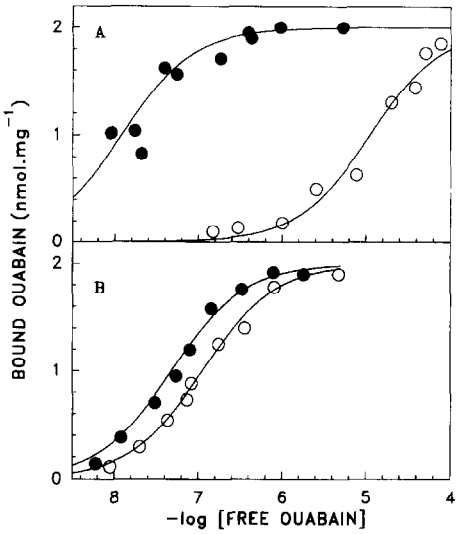

Fig. 8. Equilibrium binding of ouabain to phospho- and dephospho-forms of Na^+/K^+-ATPase in water (A) and 40% Me_2SO (B). Na^+/K^+-ATPase (35–100 μg ml^{-1}) was incubated in 1–2 ml of media containing [^{3}H]ouabain enough to obtain the free concentrations (M) given on the x-axis, 5 mM $MgCl_2$, 0.5 mM KCl, 5 mM Tris-HCl pH 7.0; zero (A) or 40% (B) Me_2SO and zero (○) or 1 mM P_i (●). After 5–8 h of incubation at 27° C, bound ouabain was measured as described in Experimental procedures. The concentration of free ouabain, always representing more than 15% of [total ouabain], was calculated as the difference between [total ouabain] and [bound ouabain] after discounting the unspecific radioactivity as described in Experimental procedures. The continuous curves were obtained by non-linear regression using an equation of binding of one ligand to one single population of sites, rendering the following dissociation constants: (A) 2 nM (●) and 11 μM (○). (B) 48 nM (●) and 112 nM (○). For all the curves the maximal binding was 2 nmol mg^{-1}, and as the phosphorylation capacity for this enzyme preparation was measured to 2.2 nmol mg^{-1}, the stoichiometry equals 1 mol of ouabain per mol phosphorylating site.

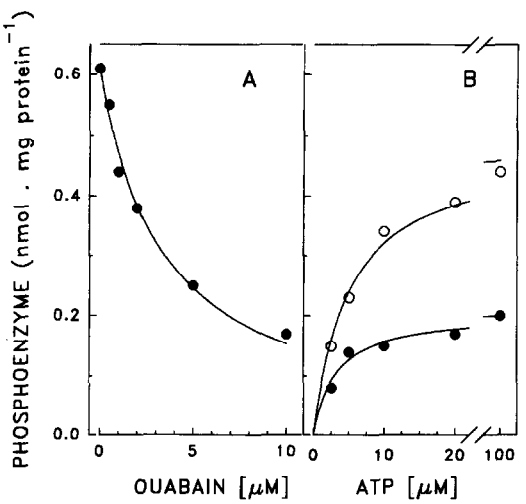

Fig. 7. Effect of ouabain on the phosphorylation from ATP in 40% Me_2SO and in the absence of Na^+. (A) Na^+/K^+-ATPase (0.1 mg ml^{-1}) was preincubated for 5 min in the Me_2SO-medium with the indicated ouabain concentrations, then 0.1 mM AT^{32}P was added and phosphorylation allowed to proceed for 1 min. (B) The same preincubation conditions as in A without ouabain (○) or with 5 μM ouabain (●). Phosphorylation was with the ATP-concentrations indicated.

4. Discussion

The results obtained in the present work indicate that the interaction of Na^+/K^+-ATPase with inorganic phosphate and ouabain in 40% Me_2SO is radically different from the similar reactions in purely aqueous media. Before discussing in detail these observations, the following points regarding the 40% Me_2SO-media should be noted: In 40% (v/v) Me_2SO the mole fractions of Me_2SO and H_2O are 0.15 and 0.85, respectively. When Me_2SO is added to aqueous protein (enzyme) solutions a gradual, reversible destabilisation of the secondary structure takes place [27,28] until a mole fraction for Me_2SO of about 0.33, equal to a solution of 65 vol% Me_2SO, is reached. At this concentration the Me_2SO-$2H_2O$ dihydrate is formed and significant changes in protein structure and also aggregation results [27,28]. In the present experiments we are well below this apparently critical level. It is also of interest, that in 40%

(v/v) Me_2SO the activity (relative to pure solvents) is 0.54 for water and 0.072 for Me_2SO. These values were calculated by Dupont and Pougeois [29] by multiplication of the relative molar concentrations with published activity coefficients for water and Me_2SO. The very low activity of Me_2SO together with the considerable reduction in water activity is a result of the pronounced interaction between the two species in the solvent mixture, and the values could be taken as support for the hypothesis that the effects observed here are mainly due to the lowering of the water activity.

Me_2SO increases the apparent affinity for inorganic phosphate of both Ca^{2+}-ATPase from the sarcoplasmic reticulum [30] and Na^+/K^+-ATPase [16,17]. For the latter enzyme k_{on} was increased and k_{off} decreased [17]. Recent studies of this effect on the Ca^{2+}-ATPase [31] suggest that the effect of Me_2SO is to favour the covalent phosphorylation (step 2, below) rather than to increase the non-covalent binding (step 1):

$$E + P_i \overset{1}{\leftrightarrow} E \cdot P_i \overset{2}{\leftrightarrow} E - P$$

The mechanism by which these Me_2SO-effects proceed is not known, but the above mentioned changes in a_{water} might play a role. Experiments, where the association constant for P_i to Ca^{2+}-ATPase was measured as a function of a_{water} in Me_2SO-water mixtures, indicated that phosphorylation at the substrate site by P_i could well be accompanied by a release of 18 water molecules [29]. The properties of the interaction of P_i with the ATPase substrate site has also been studied using an analogue of P_i, namely the inhibitor complex AlF_4^-. It was observed that increasing the Me_2SO fraction from 0 to 10% (v/v) increased the rate constant for binding of AlF_4^- to Ca^{2+}-ATPase about 20-times [32]. The quantitative relationship was compatible with the release of 22 water molecules (compare with 18 molecules found by the same authors for P_i phosphorylation, see above). In a similar study with Me_2SO and AlF_4^- binding to yeast plasma membrane H^+-ATPase [33], it was estimated from the relationship between a_{water} and the rate of onset of inhibition by AlF_4^-, that the binding process leads to release of 5 water molecules.

We may hypothesize that the lowered water activity in the Me_2SO-medium facilitates the release of water also from the substrate site of Na^+/K^+-ATPase. This could lead to a rearrangement of the functional groups that are involved both in P_i binding and in formation of the covalent P_i-aspartyl acid-anhydride bond, E-P. One might call this a transition state configuration [34,35] of the phosphorylation site where the functional groups of the protein are positioned favourably for the mentioned processes, i.e., a configuration of the substrate site which corresponds closely to that of the phosphorylated site (but in the absence of bound phosphate). This would explain the increase (by 40% Me_2SO) in apparent affinity for P_i (one could consider P_i in Me_2SO as a transition state analogue of P_i in water, relative to the enzyme configurations). It is also important in this context, that Me_2SO dramatically increases the affinity for Mg^{2+}. This is not only observed in phosphorylation studies with ATP [4] or in studies on the K^+-pNPPase activity of Na^+/K^+-ATPase [36], but also in the P_i-phosphorylation experiments relevant to this work [18].

From studies in purely aqueous media it is known that when the substrate site is phosphorylated, binding of ouabain is facilitated, i.e., the conformation of the ouabain binding site depends on the structural arrangement on the substrate site. Thus, in a 40% Me_2SO medium, where the substrate site according to the above hypothesis is in a phosphorylated conformation even in the absence of P_i, ouabain binding should be much tighter than in water (also in the absence of P_i) because the ouabain binding site is reporting the configuration of the substrate site. From Fig. 8 it is seen that K_d for ouabain, in the absence of P_i, is 11 000 nM in water and 100 nM in 40% Me_2SO. It also follows that phosphorylation in 40% Me_2SO does not result in nearly as dramatic a change in ouabain affinity as in water, see Fig. 8, since the substrate site is already in the transition state configuration (corresponding to the phosphorylated conformation) before phosphorylation. It is interesting, that changes in the substrate site without phosphorylation, leading to an increased affinity for ouabain, can be effected by an antibody directed against an epitope close to or at the substrate site [37].

Support for the above hypothesis regarding the state of the substrate site is also found in the comparison of the rate of dephosphorylation of EP and EOP in water and in Me_2SO. In the present paper we find (Fig. 4) that in 40% Me_2SO, 27° C, the dephosphorylation of EP to $E + P_i$ has a $t_{1/2}$ of about 10 s. In water this process is much faster [38], but a $t_{1/2}$ of 10 s in Me_2SO in the absence of ouabain is actually comparable to the $t_{1/2}$ for EOP → EO $+ P_i$ in water: 60 s at 0° C, 12 s at 37° C [38]. Like in water, ouabain reduces the off-rate for P_i (from EOP) in 40% Me_2SO. From Fig. 6 we might estimate a $t_{1/2}$ of about 60 min. This is comparable to the $t_{1/2}$ for release of vanadate (considered a transition state analogue of P_i – in water – which binds with a much higher affinity than P_i and promotes ouabain binding): EOV → E + O + V has a $t_{1/2} \approx 150$ min, and EV → E + V, a $t_{1/2} \approx 10$–20 min [6,39]. Since the process EO → E + O also has a $t_{1/2}$ of about 150 min [6,40], we have reason to believe that in contrast to what happens in water, where P is lost faster than ouabain from the EOP complex, P stays tightly bound in EOP in 40% Me_2SO (like vanadate in water in EOV) and that it is released only when the complex looses ouabain. In conclusion we suggest that the reduced water activity in 40% Me_2SO hardly affects the ouabain-binding site directly but that its effect on ouabain binding predominantly goes via its effect on the substrate site on Na^+/K^+-ATPase.

In our second paper in this series [4] we studied the phosphorylation of Na^+/K^+-ATPase by ATP in a 40% Me_2SO-medium similar to that used in the 'P_i-phosphorylation' experiments in the present paper. In the simplest model, that produced a satisfactory fit to the time course of phosphorylation from ATP (model C, Fig. 10 in [4]), it was necessary to have an cation independent, a priori (i.e., before addition of ATP) equilibrium between two enzyme forms E′ and E. The ratio E′/E was estimated to be around 6.25 and E, thus constituting about 14%, was the only form reacting readily with ATP. In the present paper (in the absence of ouabain) most, if not all, of the enzyme reacts rapidly with P_i (Figs. 1 and 5) and in according to model C in [4] this should then be the E′ form that would a priori constitute 86% of the enzyme [1]. This situation is not unlike what is characteristic of phosphorylation in water [2]: ATP reacts with one form (usually called E_1) and P_i 'prefers the other' (E_2), only it seems that due to the effect of Me_2SO (the decreased water activity?) on the substrate site region, the interconversion of the two conformational states of the enzyme is slow.

A problem, that we have not studied sufficiently, is the effect of Me_2SO on the cation interaction with Na^+/K^+-ATPase. It is obvious, however, that apart from interference with the phosphorylation, Me_2SO provokes changes in both Na^+ and K^+ interaction with the enzyme. Previously [3,4] we showed that phosphorylation from ATP in Me_2SO can take place in the absence of Na^+ and is stimulated by 0.5 mM K^+, and from the present study it appears that K^+ stimulates phosphorylation from P_i and that incubation of the enzyme with 100 mM Na^+ has little or no effect on ouabain binding in the presence of Mg^{2+} alone. These results are in sharp contrast to what is generally observed in aqueous media: phosphorylation by ATP requires Na^+ (for references see [41]), phosphorylation by P_i is generally observed to be inhibited by K^+ [12,17,22,46], although low concentrations, necessary to bring the enzyme into the E_2-form, are seen to stimulate [47–49]. Furthermore, Na^+ always prevent (Mg^{2+})- and ($Mg^{2+} + P_i$)-promoted ouabain binding in aqueous milieus [2].

In the above discussion we have focused on the changes in the substrate site, emphasising the similarity of ouabain binding in the absence and presence of P_i in 40% Me_2SO (and the same aspect viewed from another point – the dramatic increase in affinity for ouabain by addition of Me_2SO in the presence of Mg^{2+} alone, i.e., absence of P_i). Besides this effect of Me_2SO on the substrate site there might also be a more direct influence of lowered water activity at the ouabain binding site, indicated by the 4–10-fold increase in K_{diss} obtained when binding in water is compared with binding in 40% Me_2SO (Fig. 8) under otherwise optimal condition. The precise locus and nature of the ouabain binding site is not known, but it seems clear that the two amino acids bordering the extracellular loop connecting transmembrane segments H1 and H2 are of prime importance since, e.g., mutations of these amino acids in the sheep kidney results in a decrease of a factor of 10^2–10^3 of the ouabain affinity [42]. However, more subtle changes in affinity, i.e., 5–10-fold like those seen in the present investigation, are obtained with a number of other mutations: Schulteis et al. [43] have shown that mutation of Arg-880 to Pro, and that a number of changes in and around the H1-H2 extracellular loop (but not involving the 'border amino acids' mentioned above) in all but one case decrease the affinity 1–9-times. The latter mutations are especially interesting for this work since the amino acids were changed from residues capable of forming H-bonds to residues devoid of this property [44]. Of similar effect is the removal of the polar OH-group by the mutation of Tyr-317 to Phe [45]. It could thus be conceivable, that changes in the water structure at the ouabain-binding aspects of the ATPase are to some extent analogous to the amino acid substitutes.

[1] The question whether a small fraction of the enzyme preparation is phosphorylated and dephosphorylated slowly, Figs. 4 and 6, has not been investigated in enough detail here to warrant further discussion.

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