Effects of Organic Solvents, Methylenes, and Urea on the Affinity for P_i of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum*

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The Ca^{2+}-ATPase of sarcoplasmic reticulum can be phosphorylated by P_i, forming an acylyphosphate residue at the catalytic site of the enzyme. In a previous report (de Meis, L, Alves, E., and Martins, O. B. (1980) Biochemistry 19, 4252-4261), it was shown that organic solvent such as dimethyl sulfoxide and glycerol cause a decrease in the apparent $K_{cat}$ for P_i. In this report it is shown that a similar effect is obtained with the methylenes glycine betaine and trimethylamine N-oxide. The apparent $K_{cat}$ value for P_i in totally aqueous medium and in the presence of either 6.4 M glycerol, 1.4 M dimethyl sulfoxide, 0.4 M trimethylamine N-oxide, or 1 M glycine betaine were found to be respectively 2.85, 0.52, 0.52, 0.81, and 0.93 mM at pH 6.2 and >10.0, 1.08, 2.53, 3.05, and 2.05 mM at pH 7.5. In contrast to the effect of methylenes, urea caused an increase in the apparent $K_{cat}$ for P_i. When mixed in the appropriate concentration ratio, the effect of either organic solvent or methylenes is cancelled by urea.

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The Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles can catalyze both the hydrolysis and the synthesis of ATP (1-3). During the hydrolysis of ATP, Ca^{2+} is accumulated by the vesicles and in the reverse process, coupled with the synthesis of ATP, Ca^{2+} is released by the vesicles at a fast rate. The synthesis of ATP is initiated by phosphorylation of the ATPase by P_i, forming an acylphosphate residue at the catalytic site of the enzyme (1, 4). This reaction occurs without the need for the energy derived from the Ca^{2+} gradient (2, 3, 5). The affinity of the enzyme for P_i varies greatly with the pH of the medium (2, 5-7). The concentration of P_i needed for half-maximal enzyme phosphorylation is in the range of 2-3 mM at pH 6.0 and increases progressively to values above 10 mM as the pH of the medium is raised to the physiological values 7.2-7.4 (2, 5-7). The pH dependence of the phosphorylation reaction can be artificially abolished in vitro by adding organic solvents such as dimethyl sulfoxide or glycerol to the assay medium (8-10). These solvents promote a decrease in the enzyme's apparent $K_{cat}$ for P_i, an effect which is more pronounced at alkaline than at acidic pH values. Thus, in the presence of organic solvents, a given P_i concentration leads to the same phosphoenzyme level at pH 6.0 as at pH 7.5. Quaternary methylenes such as trimethylamine-N-oxide and glycine betaine are phylogenetically widespread compounds. Among vertebrates, they are found in the tissues of species ranging from the urea-rich marine cartilaginous fishes (sharks and rays) to mammals (11-14). In this report it is shown that these two methylenes, like organic solvents, can abolish the effect of pH on the P_i phosphorylation reaction of the Ca^{2+}-ATPase and that the effect of both methylenes and of organic solvents are antagonized by urea.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by the method of Eletr and Inesi (15). In a few experiments, vesicles loaded with calcium phosphate were prepared as previously described (6). Purification of radioactive P_i and determination of phosphoenzyme were carried out as previously described (6, 8, 16). Composition of the assay medium is described in the figure legends. In previous reports (8, 17) it was observed that organic solvents promote a small but significant change in the pH values of the different buffers used. In all experiments, the pH of the assay medium was measured in the test tube after all the reagents had been added and, when necessary, it was adjusted to the desired value. The incubation time at 35 °C was 2 min. It was previously established that equilibrium levels of phosphoenzyme are obtained after 10 s of incubation (2, 8).

Formation of the phosphoenzyme by P_i requires removal of Ca^{2+} from the medium with EGTA and no phosphoenzyme formation is observed when Ca^{2+} is added to the medium, either in the absence (2, 3, 5) or in the presence of methylenes (Fig. 1).

The pH of P_i in totally aqueous medium and in the presence of 1 M of either glycine betaine or urea was determined by titration with standardized solutions of NaOH or HCl (8, 17).

RESULTS

Effect of Methylamines—Phosphorylation of the Ca^{2+}-ATPase by P_i occurs more readily at acid than at alkaline pH (2, 5-7). In Fig. 1 the equilibrium level of phosphoenzyme was measured in the presence of 1 mM P_i. At pH 6.2 (Fig. 1A), methylamines moderately increased the phosphoenzyme level. At pH 7.6 (Fig. 1B), a considerably greater increase in the phosphoenzyme level was observed following the addition of either 0.4 M TMAO or 2 M glycine betaine to the medium. The experiment of Fig. 1 was performed using empty vesicles.

1 The abbreviations used are: EGTA, [ethylenebis(oxyethylene-nitrito)]tetraacetate acid; TMAO, trimethylamine N-oxide; MES, 2(N-morpholino)ethanesulfonic acid; EPPS, N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.
EGTA, independence of the phosphorylation reaction. It was shown in the figure and either no addition of dimethyl sulfoxide or 1 mM glycine betaine (B) were included in the water phase (8). In agreement with previous reports (8), an increase in the partition coefficient for P_i was observed when dimethyl sulfoxide was included in the water phase, but this could only be measured when the concentration of dimethyl sulfoxide was raised above 1.4 M (Table I). In totally aqueous medium and in the presence of either 0.4 M TMAO, 1.4 M dimethyl sulfoxide or 1 mM glycine betaine, the partition coefficient was too small to be measured with the method used. The data of Fig. 1 show that the effect of 0.4 M TMAO or of 1 mM glycine betaine on the phosphoryl-

Additions

<table>
<thead>
<tr>
<th>pH 6.2</th>
<th>pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.85 ± 0.30</td>
</tr>
<tr>
<td>Glycerol, 6.4 M</td>
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</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>0.52 ± 0.08</td>
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<tr>
<td>1.4 M</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>2.8 M</td>
<td>0.01</td>
</tr>
<tr>
<td>TMAO, 0.4 M</td>
<td>0.81 ± 0.15</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.93 ± 0.15</td>
</tr>
<tr>
<td>2.0 M</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td>Urea, 0.8 M</td>
<td>6.80 ± 0.30</td>
</tr>
<tr>
<td>Dimethyl sulfoxide 1.4 M plus urea 1.8 M</td>
<td>2.90 ± 0.20</td>
</tr>
<tr>
<td>Glycine betaine 1 M plus urea 1 M</td>
<td>3.10 ± 0.15</td>
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Fig. 1. Effect of methylamines on the equilibrium level of phosphoenzyme. In A the assay medium composition was 50 mM MES-Tris buffer (pH 6.2), 1 mM 32Pi, 5 mM MgCl2, and either 10 mM EGTA (O), or 1 mM CaCl2 ([ ]). In B the assay medium composition was the same except that the buffer used was EPPS (pH 7.5), 5 mM MgCl2, 10 mM EGTA, 0.35 mg of protein/ml, the 32Pi concentrations shown in the figure and either no addition (O), 0.4 M TMAO ([ ), or 1 mM glycine betaine ([ ]). Incubation and quenching were as described in Fig. 1. Double-reciprocal plots of the data obtained with TMAO and glycine betaine are shown in B.

i.e. in the absence of a transmembrane Ca2+ gradient. At pH 7.5 essentially the same degree of activation by glycine betaine as that obtained with empty vesicles was observed when vesicles previously loaded with calcium phosphate (Ca2+ gradient) were used (data not shown). Both glycine betaine and TMAO led to a decrease in the apparent K_m for P_i. This effect was more pronounced at alkaline than at acidic pH values (Fig. 2 and Table I). As previously described (8), a similar effect is observed when one of the organic solvents glycerol or dimethyl sulfoxide is added to the medium (Table I). The effects of methylamines and organic solvents were additive (Fig. 3). This could be observed clearly in the presence of low concentrations of organic solvent. The maximal number of sites available to be phosphorylated by P_i in sarcoplasmic reticulum vesicles is in the range of 3.5-4.5 μmol/g of protein. In the presence of a large concentration of dimethyl sulfoxide, all the catalytic sites of the enzyme are phosphorylated by P_i, and the effect of adding methylamine can no longer be detected. The double reciprocal plot of Fig.

Fig. 2. Effect of methylamines on the P_i concentration dependence of the phosphorylation reaction. The assay medium composition was 50 mM EPPS buffer (pH 7.5), 5 mM MgCl2, 10 mM EGTA, 0.35 mg of protein/ml, the 32Pi concentrations shown in the figure and either no addition (O), 0.4 M TMAO ([ ), or 1 mM glycine betaine ([ ]). Incubation and quenching were as described in Fig. 1. Double-reciprocal plots of the data obtained with TMAO and glycine betaine are shown in B.

Fig. 3. Effect of dimethyl sulfoxide on the equilibrium level of phosphoenzyme in the absence and in the presence of methylamines. The assay medium composition was 50 mM EPPS buffer (pH 7.5), 1 mM 32Pi, 5 mM MgCl2, 10 mM EGTA and either no addition (O), 0.4 M TMAO ([ ), or 1 mM glycine betaine ([ ]). In the figure, Me2SO refers to dimethyl sulfoxide. Other conditions were as in Fig. 1. 2B shows that the maximal level of phosphorylation was not affected by methylamines.

Partition Coefficient—Previous evidence (8, 9, 16-23) indicates that the catalytic site of the Ca2+-ATPase is hydrophobic when the enzyme is in the conformation that is phosphorylated by P_i. These results have been interpreted to mean that different organic solvents facilitate the partition of P_i from the assay medium into the catalytic site of the enzyme by decreasing the difference of hydrophobicity between these two compartments. This would promote an increase in the enzyme affinity for P_i. Supporting this view, it was found that the partition of P_i between a water phase (assay medium) and an organic phase containing benzene and isobutyl alcohol increased when cosolvents such as dimethyl sulfoxide and glycerol were included in the water phase (8). In agreement with previous reports (8), an increase in the partition coefficient for P_i was observed when dimethyl sulfoxide was included in the water phase, but this could only be measured when the concentration of dimethyl sulfoxide was raised above 1.4 M (Table II). In totally aqueous medium and in the presence of either 0.4 M TMAO, 1.4 M dimethyl sulfoxide or 1 mM glycine betaine, the partition coefficient was too small to be measured with the method used. The data of Fig. 3 show that the effect of 0.4 M TMAO or of 1 mM glycine betaine on the phosphoryl-
phosphate (H_2PO_4^-) is able to phosphorylate the ATPase. The effect of urea and dimethyl sulfoxide on the partition coefficient of P_i

<table>
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<th>Additions</th>
<th>Organic-aqueous phase partition coefficient</th>
</tr>
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<tbody>
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<td>None</td>
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</tr>
<tr>
<td>TMAO, 0.4 M</td>
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</tr>
<tr>
<td>Glycine betaine, 2 M</td>
<td>ND</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>ND</td>
</tr>
<tr>
<td>1.4 M</td>
<td>ND</td>
</tr>
<tr>
<td>2.8 M</td>
<td>5.04 ± 1.34 x 10^-9</td>
</tr>
<tr>
<td>4.2 M</td>
<td>1.41 ± 0.37 x 10^-9</td>
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<td>Dimethyl sulfoxide 2.8 M plus 1 M NH_4Cl</td>
<td>4.72 ± 1.27 x 10^-9</td>
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<tr>
<td>Dimethyl sulfoxide 2.8 M plus 1 M urea</td>
<td>ND</td>
</tr>
<tr>
<td>Dimethyl sulfoxide 2.8 M plus 3 M urea</td>
<td>ND</td>
</tr>
</tbody>
</table>

The pK_a of P_i-Beil et al. is able to phosphorylate the ATPase. Thus, changes in apparent K_a for P_i observed after the addition of methylamines and urea to the assay medium could be related solely to an effect of these compounds on the pK_a of P_i. The partition coefficient was calculated by dividing the concentration of P_i in dimethyl sulfoxide-water mixtures by its concentration in the aqueous phase.

**Discussion**

**Apparent K_a for P_i**

The Ca^{2+}-ATPase undergoes a conformational change during the catalytic cycle (for reviews see 2, 7).
In one of its conformations (E) the enzyme has a high affinity for Ca\(^{2+}\) and is phosphorylated by ATP but not by Pi. In the second conformation (E'), the enzyme has a low affinity for Ca\(^{2+}\) and is phosphorylated by Pi, but not by ATP. The experiments presented in this report were performed in the absence of ATP and Ca\(^{2+}\). In this condition the two enzyme forms coexist in equilibrium and the phosphorylation by Pi is described as:

\[
E \rightleftharpoons E' \rightleftharpoons E\cdot Pi \rightleftharpoons E - Pi + H_2O
\]

At pH 6.0–6.5, the equilibrium constant of reaction 1 (*E/E) is about one (2, 25), and it decreases as the pH of the medium is raised. Thus, at pH 7.4 practically all the enzyme is in the form E (2). The equilibrium of reaction 3 strongly favors the formation of the phosphoenzyme (8, 26). Therefore, a small change in the apparent K_m of reaction 2 can lead to a significant change in the apparent K_m for Pi, (8). Experiments described in previous reports (8, 9, 16–23) indicate that the catalytic site of the enzyme form *E is hydrophobic. Dupont and Pougeois (10) suggested that the binding of Pi to the Ca\(^{2+}\)-ATPase promotes a conformational change of the protein which would lead to the release of a large number of water molecules from the active site. In this view, the active site of the enzyme would only become hydrophobic after the binding of Pi. This proposal is not supported by the findings obtained recently in different laboratories. Solvent accessibility studied by fluorescence quenching (21, 22) and hydrophobic labeling of the Ca\(^{2+}\)-ATPase with trifluoromethyl-iodophenyl-diazirine (23) indicate that the catalytic site of the enzyme form *E had the hydrophobic character before the addition of Pi and that the binding of Pi did not promote a detectable change of polarity or water accessibility in the catalytic site. The finding that organic solvents increase the partition coefficient of Pi, from an aqueous phase into an organic phase (Ref. 8 and Table I) indicates that dimethyl sulfoxide changes the properties of the solution (assay medium). We infer that this change is responsible for changes of the K_m of reaction 2 and a decreased apparent K_m for Pi. The partition coefficient data obtained with methylamines were not sufficient to distinguish whether the decrease in the apparent K_m for Pi, was promoted by a change in the K_m of reaction 1, 2, or 3. The finding that the effect of methylamines is small at pH 6.0 and pronounced at pH 7.5 may indicate that they change the K_m of reaction 1. On the other hand, the finding that urea abolishes the effect of dimethyl sulfoxide on the partition coefficient of Pi, and that it also abolishes the effect of dimethyl sulfoxide and methylamines on the K_m for Pi, suggests that like organic solvents, the methylamines may promote a change in the K_m of reaction 2. More experimentation is required to untangle this and other possibilities.

### Physiological Role

Methylamines have been observed in different types of tissues for many years; however, their physiological role is still unknown (13, 27, 28). Recently it has been proposed that methylamines may serve to protect certain cells from the toxic effect of urea by counteracting its effect on protein structures (11, 14). Concentrations of urea varying from 0.4–0.6 M occur commonly in the mammalian kidney and throughout the tissues of carangidous fishes (11, 14). In these fishes the high concentration of urea serves to balance the high osmolarity of the sea water. Wilkie and Wray (28) measured the concentration of the methylamine glycerophosphorylcholine in the gastrocnemius muscles of frogs by 31P NMR spectroscopy. They observed a simultaneous increase in blood urea and in the concentration of the methylamine in muscle when frogs kept in tap water were exposed to water made hypertonic (300 mosmol/liter) with NaCl. Vancz and Somero (11, 14) reported that the activity of argininosuccinate lyase from pig kidney or from liver of the thornback ray is noncompetitively inhibited by 0.4 M urea, and that this effect is abolished when 0.2 M TMAO is added to the assay medium together with urea. Ca\(^{2+}\) transport ATPases with properties similar to that found in the sarcoplasmic reticulum have been described in brain, red cells, liver, platelets, and kidney (29–36). Using 31NMR, Balaban and Knepper (13) estimated that the intracellular concentration of methylamine compounds in rat and rabbit kidney is near 200 mmol/kg of intracellular water. At these concentrations their effects on the phosphorylation of the Ca\(^{2+}\)-ATPase by Pi are not maximal, but in the physiological pH range, they are not trivial. In Fig. IB, for example, the phosphoenzyme formation doubled in the presence of 0.1 M TMAO or 0.2 M glycine betaine. Therefore, besides protecting the cell from the toxic effect of urea, perhaps methylamines may also be involved in regulating the affinity for Pi, of the Ca\(^{2+}\)-ATPase.

### Effect of Organic Solvent on Different Enzymes

In addition to their effects on the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum, organic solvents have been found to promote a decrease in the apparent K_m for Pi, of different enzymes involved in processes of energy transduction. These include the soluble F_1-ATPase of mitochondria (37–41), yeast inorganic pyrophosphatase (42), and the H^-ATPase of yeast plasma membrane (43). There are large structural differences between the molecules of these different enzymes. The Ca\(^{2+}\)-ATPase has only one type of subunit, whereas the F_1-ATPase is an oligomer containing several different subunits. The Ca\(^{2+}\)-ATPase, the (Na + K^-ATPase and the H^-ATPase of yeast are membrane-bound enzymes, whereas the F_1 of mitochondria and the yeast pyrophosphatase are water-soluble enzymes without bound lipids. These differences suggest that the decrease in the apparent K_m for Pi, is not related to a specific interaction of the organic solvent with the enzyme, but rather to a change of water activity promoted by the cosolvent. The water molecules that organize around a protein in solution have properties that are different from those of medium "bulk water," for example a lower vapor pressure, a lower water mobility, and a greatly reduced freezing point (44, 45). Similar changes in the properties of water are observed in mixtures of organic solvents and water (46, 47). At present we do not know the structure of water in the different compartments of the cell. For instance, because of the very high protein concentration found in the mitochondrial matrix (48, 49), it is unlikely that in physiological conditions the solvent structure in this organelle is the same as that of the aqueous solutions usually used for experiments in vitro. The data presented raise the possibility that methylamines and urea may play a physiological role in modulating the affinity for Pi, of the Ca\(^{2+}\)-ATPase and perhaps, of other enzymes involved in processes of energy transduction. In the cell the different methylamines would modify the properties of bulk water in a manner similar to that observed with the use of organic solvents, and urea would antagonize this effect.

### REFERENCES

Methylamines and Sarcoplasmic Reticulum ATPase