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# Role of water activity on the rates of acetyl phosphate and ATP hydrolysis

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The rates of hydrolysis of acetyl phosphate in the presence of 0.1 M NaOH and of ATP in the presence of either 1 M HCl or 1 M NaOH were measured at different temperatures and in the presence of different concentrations of the organic solvents dimethyl sulfoxide or ethylene glycol. Under all conditions tested, there was a progressive increase in the rate constant of hydrolysis of both phosphate compounds as the water activity of the medium was decreased by the addition of organic solvents. At 25°C, substitution of 70% of the water of the medium by dimethyl sulfoxide promoted an increase of two orders of magnitude in the rate constant of acetyl phosphate hydrolysis. In the presence of 80% and 90% dimethyl sulfoxide the rate of acetyl phosphate hydrolysis increased by more than two orders of magnitude and was so fast that it could not be measured with the method used. The effect of organic solvents on the rate of ATP hydrolysis was less pronounced than that observed for acetyl phosphate hydrolysis. At 30°C, substitution of 90% of water by an organic solvent promoted a 4–6-fold increase of the rate of ATP hydrolysis. Acceleration of either acetyl phosphate or ATP hydrolysis rates was promoted by a decrease in both activation energies ($E_a$) and in entropies of activation $\Delta S\ddagger$. The data obtained are discussed with reference to the mechanism of catalysis of enzymes involved in energy transduction such as the $Ca^{2+}$-ATPase of sarcoplasmic reticulum and the $F_1$-ATPase of mitochondria.

Catalysis; ATP; Acyl phosphate

## 1. INTRODUCTION

In water the hydrolysis of an acyl phosphate residue, ATP or inorganic pyrophosphatase is accompanied by a large change in free energy [2,3]. The energy of hydrolysis of these phosphate compounds can vary greatly during the catalytic cycle of enzymes that cleave them. In the initial steps of the cycle the compound has a high energy of hydrolysis, but in subsequent steps only a small energy change occurs. The decrease in energy of hydrolysis of the phosphate compounds that occurs on the enzyme surface appears to be crucial to the mechanism of energy transduction in the living cell. Among the enzymes in which this phenomenon is observed are the $Ca^{2+}$-ATPase of sarcoplasmic reticulum [4–7], the (Na+K)-ATPase of plasma membranes [8], $F_1$-ATPase of mitochondria and chloroplasts [9–12], myosin [13] and inorganic pyrophosphatase [14,15]. A common feature of these enzymes is that they undergo a conformational change during the catalytic cycle. Recent evidence [7,16–28] indicates that the conformational change leads to a decrease in the water activity within the catalytic site and that this hydrophilic-hydrophobic transition causes a decrease in the energy of hydrolysis of the phosphate compound. The phosphate compound would bind to the enzyme when the catalytic site is hydrophilic; once bound it would remain a high energy molecule but would not be hydrolyzed. Following the conformational change, the catalytic site would become hydrophobic, there would be a large decrease in the equilibrium constant ($k_{eq}$) for hydrolysis and then the phosphate compound would be cleaved. An apparent paradox in this

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*Abbreviations:* $Me_2SO$, dimethyl sulfoxide; $k_r$, rate constant for hydrolysis

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proposal is that the phosphate compound is hydrolyzed when the catalytic site becomes hydrophobic. Because water is one of the reactants for the hydrolytic reaction, one would expect that a decrease in water activity should impair the cleavage of the phosphate compound and not facilitate it. In this report we measured the spontaneous hydrolysis of acetyl phosphate and ATP in alkaline and acid solutions. The water activity of the system was decreased by adding different amounts of organic solvent to the media. The aim was to simulate in the test tube a possible effect of water activity in the mechanism of catalysis that normally occurs on the enzyme surface.

## 2. EXPERIMENTAL

$^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and purified as a phosphomolybdate complex with a mixture of benzene and isobutyl alcohol [19]. [$\gamma$-$^{32}$P]ATP was prepared according to the method of Glynn and Chappell [29]. ATP hydrolysis was assayed by measuring the release of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP. The reaction was started by adding [$\gamma$-$^{32}$P]-ATP and stopped by removing the ATP not hydrolyzed from the mixture with charcoal [30]. A 0.1 ml sample of the medium was diluted in 0.4 ml of an ice-cold suspension of 25% charcoal in 0.1 M HCl. The sample was centrifuged for 10 min in the cold at 5000 × $g$ and a sample of each supernatant was counted in a scintillation counter. In each experiment controls were performed in which the radioactive ATP was added to the mixture 1 or 2 s after the addition of the charcoal suspension. Acetyl phosphate hydrolysis was assayed by measuring the unhydrolyzed acetyl phosphate [31]. The reaction was started by adding acetyl phosphate to the medium and arrested by diluting a 0.2 ml sample in 1.05 ml of an ice-cold acetic acid solution. The concentration of acetic acid was adjusted to produce in the final mixture a pH value in the range of 5.0–6.0. This was followed by the addition of 0.1 ml to 0.5 M acetate buffer (pH 5.4) and 0.25 ml of a 22.7% (w/v) hydroxylamine solution adjusted to pH 6.4 with NaOH. This mixture was incubated for 15 min at room temperature before addition of 0.5 ml of 2.5 M HCl, 0.5 ml of a 12% (w/v) trichloroacetic acid solution and 0.5 ml of a freshly prepared solution of 14.7% (w/v) $FeCl_3$ in 0.1 M HCl. Optical absorbance was measured at 520 nm. In each experiment, controls were performed in which the acetyl phosphate was added after the addition of the ice-cold acetic acid quenching solution.

The concentrations of $Me_2SO$ and of ethylene glycol used did not interfere with the methods used for measuring ATP and acetyl phosphate hydrolysis.

## 3. RESULTS

### 3.1. *Acetyl phosphate hydrolysis*

In the presence of 0.1 M NaOH the rate of acetyl phosphate hydrolysis increased when either $Me_2SO$ or ethylene glycol was added to the medium (fig.1A). There was a progressive increase in $k$ as the water activity of the medium was decreased by raising the concentration of $Me_2SO$ (fig.2A). The $E_a$ and $\Delta S^{\ddagger}$ were calculated from an Arrhenius plot of $k$ measured at different temperatures (fig.2B). In totally aqueous media both $E_a$ and $\Delta S^{\ddagger}$ were high and positive, and decreased when $Me_2SO$ was added to the medium (fig.2C and table 1). There was a linear relationship between the decrease in both $E_a$ and $\Delta S^{\ddagger}$ and the decrease in water activity promoted by $Me_2SO$ (fig.2C). In the presence of $MgCl_2$, concentrations of organic solvents higher than those shown in fig.2 and table 1 were not used in order to avoid the formation of insoluble magnesium-acetyl phosphate complexes. In the absence of magnesium, the rate of hydrolysis of acetyl phosphate was measured in mixtures containing up to 90% $Me_2SO$. As in the experiments with magnesium, the rate constant increased with higher concentrations of $Me_2SO$ and in the presence of 80% and 90% $Me_2SO$, the rate of hydrolysis was so fast that it could not be measured with the method used: practically all the acetyl phosphate being hydrolyzed in less than 5 s (table 1).

In mixtures of water and organic solvent the molar fraction of water becomes smaller, the higher the concentration of organic solvent used. Thus the decreases of $E_a$ and $\Delta S^{\ddagger}$ observed in fig.2C could be related to an increase in the molar

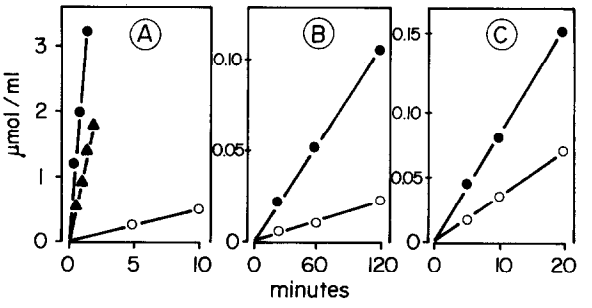


Fig.1. Hydrolysis of acetyl phosphate and of ATP. In A, the media contained 0.1 M NaOH, 5 mM acetyl phosphate and either no additions (○) or 50% (v/v) of either ethylene glycol (▲) or $Me_2SO$ (●). The temperature was 25°C. In B, the media contained 1 M NaOH, 10 mM $MgCl_2$, 1 mM ATP and either no additions (○) or 50% (v/v) $Me_2SO$ (●). The temperature was 30°C. In C, the media contained 1 M HCl, 1 mM ATP and either no additions (○) or (●) 50% (v/v) $Me_2SO$. The temperature was 35°C.

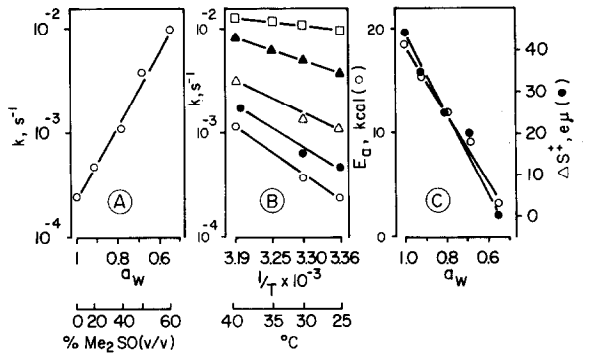


Fig.2. Hydrolysis of acetyl phosphate in the presence of magnesium. Hydrolysis was measured in media containing 0.1 M NaOH, 5 mM acetyl phosphate, 10 mM $MgCl_2$ and 0–60% $Me_2SO$. In A, the temperature was 25°C. The water activities ($a_w$) corresponding to the different $Me_2SO$ concentrations used were determined from vapor pressure values [35]. In B, the concentrations of $Me_2SO$ were (○) 0, (●) 20%, (△) 40%, (▲) 50% and (□) 60%. Other conditions as in A. (C) Activation energies (○) and entropies of activation (●) calculated from the Arrhenius plot shown in B.

ratio $HO^-$/HOH and not to decreased water activity. This possibility is excluded by the finding that in totally aqueous media, raising the NaOH concentration from 0.1 to 0.5 M promoted a small increase of $\Delta S^{\ddagger}$ and had no effect on $E_a$ (table 1).

### 3.2 *ATP hydrolysis*

The effect of organic solvents on the rate of ATP hydrolysis was less pronounced than that observed for acetyl phosphate hydrolysis. ATP was hydrolyzed at a slower rate in the presence of 1 M NaOH (fig.1B) than in the presence of 1 M HCl (fig.1C). At both acidic and alkaline pH values, the rate of hydrolysis increased when $Me_2SO$ was included in the assay media (fig.1B and C). As for acetyl phosphate, in totally aqueous media $E_a$ and $\Delta S^{\ddagger}$ had high positive values which decreased when either $Me_2SO$ or ethylene glycol was present (fig.3 and table 1). Essentially the same values of $E_a$ and $\Delta S^{\ddagger}$ were obtained using either 0.2 or 1.0 M HCl. This was observed both in totally aqueous media and in the presence of 90% $Me_2SO$ (table 1). In the presence of NaOH, the concentration of organic solvent could not be raised above 50% because ATP becomes insoluble both in the presence and in the absence of magnesium.

### 3.3 *Effect of magnesium on the rates of acetyl phosphate and ATP hydrolysis*

In a totally aqueous medium the addition of $MgCl_2$ promoted a decrease in the rate constant for

Table 1

Effect of organic solvents on the hydrolysis of acetyl phosphate and ATP

| Additions | $k$ ($s^{-1}$) | $E_a$ (kcal) | $\Delta S^{\ddagger}$ (e.u.) |
|---|---|---|---|
| Acetyl phosphate (5 mM) | | | |
| 0.1 M NaOH | $4.8 \times 10^{-4}$ | 10.7 | 18.8 |
| 0.5 M NaOH | $7.6 \times 10^{-4}$ | 10.6 | 22.8 |
| 0.1 M NaOH + 70% $Me_2SO$ | $3.2 \times 10^{-2}$ | 4.1 | 4.5 |
| 0.1 M NaOH + 90% $Me_2SO$ | $>2.0 \times 10^{-1}$ | - | - |
| 0.1 M NaOH + 10 mM $MgCl_2$ | $2.5 \times 10^{-4}$ | 18.5 | 43.7 |
| 0.1 M NaOH + 10 mM $MgCl_2$ + 60% $Me_2SO$ | $10^{-2}$ | 3.3 | 0.1 |
| ATP (1 mM) | | | |
| 1 M NaOH | $1.3 \times 10^{-8}$ | 27.9 | 58.9 |
| 1 M NaOH + 50% $Me_2SO$ | $4.1 \times 10^{-8}$ | 19.5 | 37.0 |
| 1 M NaOH + 10 mM $MgCl_2$ | $3.0 \times 10^{-6}$ | 17.0 | 28.3 |
| 1 M NaOH + 10 mM $MgCl_2$ + 50% $Me_2SO$ | $1.8 \times 10^{-5}$ | 10.7 | 11.5 |
| 1 M NaOH + 10 mM $MgCl_2$ + 50% ethylene glycol | $1.4 \times 10^{-5}$ | 11.4 | 13.2 |
| 1.0 M HCl | $5.8 \times 10^{-5}$ | 17.9 | 37.7 |
| 1.0 M HCl + 90% $Me_2SO$ | $2.4 \times 10^{-4}$ | 13.5 | 25.5 |
| 0.2 M HCl + 90% $Me_2SO$ | $1.8 \times 10^{-4}$ | 14.2 | 29.4 |
| 1.0 M HCl + 90% ethylene glycol | $1.4 \times 10^{-4}$ | 14.2 | 29.4 |

Experimental conditions were as described in figs 1–3. The rate constants for the hydrolysis ($k$) of acetyl phosphate were measured at 25°C, and those for ATP hydrolysis were at 30°C

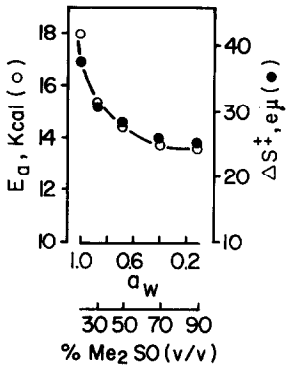


Fig.3. ATP hydrolysis in the presence of HCl. The medium contained 1 M HCl, 1 mM ATP and the concentrations of $Me_2SO$ shown in the figure. Rates of hydrolysis were measured at 30, 35, 40, 45 and 50°C.

acetyl phosphate hydrolysis and an increase in both $E_a$ and $\Delta S^{\ddagger}$ (table 1). Opposite results were obtained in a totally aqueous medium with the use of ATP. In the presence of NaOH, the addition of $MgCl_2$ led to an increase in the hydrolysis rate and a decrease in both $E_a$ and $\Delta S^{\ddagger}$ (table 1). Using a 1 mM ATP solution in 1 M NaOH it was found that the rate of hydrolysis increased as the magnesium concentration was raised, reaching a maximum in the presence of 5 mM $MgCl_2$ that did not change when the $MgCl_2$ was increased to 10 mM (not shown). Both in the presence and absence of magnesium, the addition of organic solvents to the medium promoted a decrease in $E_a$ and $\Delta S^{\ddagger}$. The differences between values obtained with and without magnesium decreased in the presence of organic solvent.

## 4. DISCUSSION

In mixtures of organic solvents with water, the observed energy of hydrolysis of acetic anhydride [26] and of phosphoric anhydrides such as pyrophosphate and ATP [19,20,26] is 5–10 kcal smaller than that measured in totally aqueous media. The data presented show that a decrease in water activity in the medium promotes an increase in the rate of hydrolysis of phosphate compounds in solution. Extrapolated to enzymes involved in energy transduction, these data might indicate that in addition to decreasing the energy of hydrolysis, a decrease in water activity within the catalytic site can accelerate the rate of hydrolysis of the phosphate compound. This dual effect can be explained if the decrease in water activity promotes an increase in the rate constants for both synthesis and hydrolysis of the compound, with the rate constant for synthesis being accelerated more than that for hydrolysis.

The catalytic cycles of both the $Ca^{2+}$-ATPase of sarcoplasmic reticulum and the (Na + K)-ATPase of plasma membranes include two distinct functional states of the enzyme, $E_1$ and $E_2$ [6,7,32–34]. Depending on the enzyme, the form $E_1$ binds either $Ca^{2+}$ or $Na^+$ with high affinity on one side of the membrane (fig.4). Following the binding of the cation, an aspartyl residue located in the catalytic site of each enzyme is phosphorylated by ATP, forming an acyl phosphate residue (reaction 1). The cation is translocated across the membrane during the conversion of the phosphoenzyme form $E_1$-P into $E_2$-P (reaction 2). After this conversion there is a large decrease in the enzyme affinity for the cation, which permits its dissociation on the other side of the membrane, and a large decrease in the energy of hydrolysis of the acyl phosphate residue (reaction 3). The hydrolysis of the enzyme forms $E_1$-P occurs at much slower rates than that of the enzyme forms $E_2$-P. If this were not the case, the hydrolysis of ATP would not be accompanied by the translocation of the cation through the membrane. The mechanism which determines the difference in the rate of hydrolysis of $E_1$-P and $E_2$-P is not known. The data presented in this report may indicate that it could be related to a decrease of water activity in the catalytic site of the enzyme. As for acetyl phosphate in totally aqueous medium, the $E_a$ and $\Delta S^{\ddagger}$ of hydrolysis of $E_1$-P would have a high positive value because of the high water activity in the catalytic site. After the conformational change of the phosphoenzyme (reaction 2 in fig.4) there would be a decrease in water activity in the catalytic site and this would allow the $E_2$-P to be hydrolyzed at a faster rate due to a decrease in both $E_a$ and $\Delta S^{\ddagger}$, as observed for acetyl phosphate in the presence of organic solvents (figs 1A and 2).

The same mechanism might also apply to the $F_1$-ATPase of mitochondria, which spontaneously forms 'tightly bound' ATP from ADP and $P_i$ [9–12], and to yeast inorganic pyrophosphatase which forms 'tightly bound' pyrophosphate from $P_i$ [14,15]. In these enzymes two different forms of

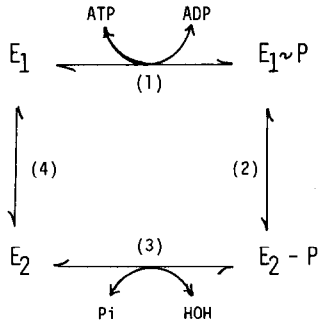


Fig.4. Reaction sequence.

enzyme-substrate complex are formed in sequence during the catalytic cycle. In one of them the substrate has a high energy of hydrolysis, dissociates easily from the enzyme and is hydrolyzed at a slow rate. In the second, the substrate has a low energy of hydrolysis, does not dissociate from the enzyme (i.e., tightly bound) and is hydrolyzed at a fast rate. In previous reports it has been shown that a decrease in water activity promotes a significant decrease in the energy of hydrolysis of both pyrophosphate and of ATP [19,20,26]. This and the data of figs 1 and 3 raise the possibility that the difference in properties of the two forms of enzyme-substrate complexes could be related to a decrease in water activity at the catalytic site. As for the $E_1$-P and $E_2$-P phosphoenzymes, the water activity in the catalytic site of the 'loosely bound' enzyme-substrate complex would be higher that that of the 'tightly bound' complex.

Finally, at present we do not know why a decrease of water activity promotes a decrease in the $E_a$ and $\Delta S^{\ddagger}$ of both acetyl phosphate and ATP hydrolysis.

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