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Energy transduction at the catalytic site of enzymes: hydrolysis of phosphoester bonds and synthesis of pyrophosphate by alkaline phosphatase

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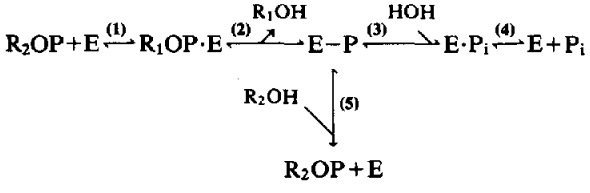
Alkaline phosphatase from mouse intestinal epithelial cells catalyzes the synthesis of pyrophosphate from P_i during hydrolysis of either glucose 6-phosphate, ATP, ADP, inorganic pyrophosphate or *p*-nitrophenylphosphate. The rate of pyrophosphate synthesis is increased by $MgCl_2$ and by decreasing the pH of the medium from 8.5 to 6.0. The data presented indicate that at the catalytic site of alkaline phosphatase the energies of hydrolysis of the phosphoserine residue and of pyrophosphate are different from those measured in aqueous solutions.

Energy transduction; Catalytic site; Alkaline phosphatase

1. INTRODUCTION

The energy of hydrolysis of the phosphoanhydride bonds of ATP, pyrophosphate and acyl phosphate residues varies greatly depending on whether these compounds are in solution or bound to the catalytic site of enzymes (for review see [1]). Water activity seems to play a major role in determining the energy of hydrolysis of these compounds [1-4]. Recently [5] it has been shown for phosphoester bonds such as phosphoserine and glucose phosphate that, different from phosphoanhydride bonds, the energy of hydrolysis does not vary when the water activity of the medium is altered. In a totally aqueous medium, the ΔG° of pyrophosphate hydrolysis is 2-3 kcal/mol more negative than the ΔG° for hydrolysis of either phosphoserine or glucose phosphate [5-8]. This means that the energy derived from the hydrolysis of a phosphoserine molecule is not sufficient to promote the synthesis of pyrophosphate from two molecules of P_i. However, in the presence of organic solvents this situation is reversed and the ΔG° of either phosphoserine or glucose phosphate becomes more negative than that of pyrophosphate [5]. In this situation the hydrolysis of phosphoserine is sufficient to promote the synthesis of pyrophosphate.

Alkaline phosphatase catalyzes the hydrolysis of both phosphoanhydride and phosphoester bonds [9]. The catalytic cycle of the enzyme can be represented as follows [9-12]:



Hydrolysis of either phosphoanhydrides or phos-

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid

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phoesters is initiated by phosphorylation of a serine residue located at the catalytic site of the enzyme (reactions 1 and 2). In the next step, the phosphoseryl intermediate is hydrolyzed, leading to the production of P_i (reactions 3 and 4). Alternatively, the phosphoseryl intermediate can transfer the phosphate to any of a large number of acceptor alcohols (reaction 5). Thus, during the hydrolysis of one phosphoester, the enzyme can synthesize another one [12]. Because there is a large difference in the energy of hydrolysis between phosphoester and phosphoanhydride bonds in water, it was thought that the intermediate phosphoserine could transfer the phosphate to an alcohol but could not form a phosphoanhydride bond. In this report it is shown that alkaline phosphatase can catalyze the synthesis of pyrophosphate.

2. MATERIALS AND METHODS

2.1. *Alkaline phosphatase*

Alkaline phosphatase (EC 3.1.3.1) was purified from mouse intestinal epithelial cells by butanol solubilization, alcohol fractionation, and molecular-sieve chromatography, followed by affinity chromatography with L-histidyl diazobenzylphosphonic acid agarose gel [13,14].

2.2. *Pyrophosphate synthesis*

Synthesis of pyrophosphate was assayed using $^{32}P_i$ obtained from the Brazilian Institute of Atomic Energy and purified as previously described [2]. The reactions were quenched with trichloroacetic acid and unreacted P_i was extracted as a phosphomolybdate complex with isobutyl alcohol/benzene [2,3]. In two different experiments the radioactive material formed during incubation was isolated and characterized in order to confirm that it was pyrophosphate. The experiments were performed in a large volume of assay medium (2 ml) and the conditions were as described in figs 1A and 2 using either glucose 6-phosphate or glucose 6-phosphate plus ADP or ATP as substrates. The reactions were quenched after 2 min (glucose 6-phosphate plus ADP) or 5 min (ATP and glucose 6-P). Then the $^{32}P_i$ was extracted and the radioactive material remaining in solution was precipitated, isolated and solubilized as previously described [3]. Recovery of the radioactive material after isolation varied between 89 and 92%. Autoradiography of ascending thin-layer chromatograms [3,15] performed using 0.75 M KH_2PO_4 adjusted to pH 3.4 with phosphoric acid revealed that the radioactive material had the same R_f as inorganic pyrophosphate. After treatment with inorganic pyrophosphatase the material no longer moved with the same R_f as pyrophosphate and a single radioactive spot moving at the front of the chromatogram with the same R_f as control $^{32}P_i$ was detected.

2.3. *ATPase activity*

ATPase activity was assayed by measuring the release of P_i from [γ-^{32}P]ATP [16]. The hydrolysis of pyrophosphate, ADP, glycerol phosphate and glucose 6-phosphate was assayed by measuring the release of P_i colorimetrically [17]. The hydrolysis of *p*-nitrophenylphosphate was assayed by measuring the formation of *p*-nitrophenyl colorimetrically at 405 nm [14].

3. RESULTS

Synthesis of pyrophosphate from $^{32}P_i$ added to the medium was detected during the hydrolysis of either glucose 6-phosphate, ADP or ATP (figs 1 and 2). The rate of substrate hydrolysis was about 1000 times faster than the rate of pyrophosphate synthesis. Notice that different units are used to express the rates of synthesis (figs 1A and 2) and hydrolysis (figs 1B and 2). With all substrates, the amount of pyrophosphate synthesized from added $^{32}P_i$ increased as a function of time and then decreased as the substrate included in the assay medium was consumed. This indicates that the radioactive pyrophosphate synthesized from the added $^{32}P_i$ was also hydrolyzed by the enzyme.

That the radioactive material formed in figs 1A and 2 was indeed pyrophosphate was confirmed using two different experimental approaches. In one of them yeast inorganic pyrophosphatase was included in the assay medium together with alkaline phosphatase. After excess $^{32}P_i$ was removed by ex-

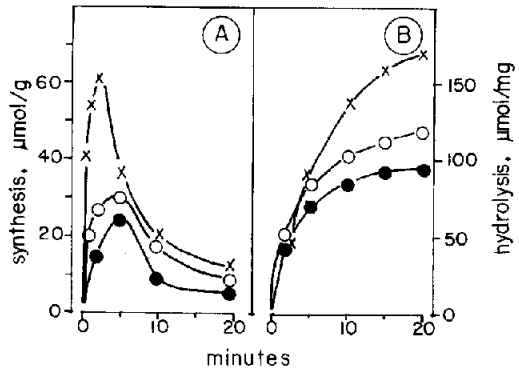

Fig.1. Synthesis of pyrophosphate (A) during hydrolysis of glucose 6-phosphate and ADP (B). Composition of the assay medium was 40 mM Mes-KOH buffer (pH 6.0), 12 mM $MgCl_2$, 4 mM of either $^{32}P_i$ (A) or non-radioactive P_i (B) and 4 mM glucose 6-phosphate (●), 4 mM ADP (○) or 4 mM glucose 6-phosphate plus 4 mM ADP (×). The reaction was started by the addition of enzyme to a final concentration of 40 µg/ml and arrested after different incubation intervals at 35°C by the addition of trichloroacetic acid to a final concentration of 10% (w/v).

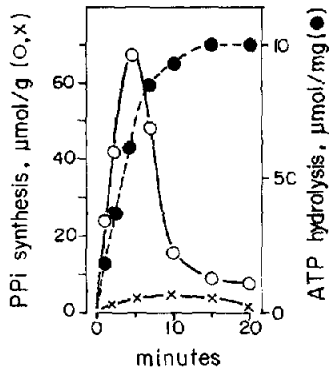

Fig.2. Synthesis of pyrophosphate measured during the hydrolysis of ATP. Composition of the assay medium was 40 mM Mes-KOH buffer (pH 6.0), 12 mM $MgCl_2$, 4 mM P_i and 4 mM ATP, either without (○,●) or with (×) 20 μg/ml bakers' yeast inorganic pyrophosphatase. The reaction was started by the addition of alkaline phosphatase to a final concentration of 40 μg/ml and arrested after different incubation intervals at 35°C by the addition of trichloroacetic acid to a final concentration of 10% (w/v). For the synthesis of pyrophosphate (○,×), $^{32}P_i$ and non-radioactive ATP were used. For the hydrolysis of ATP (●), non-radioactive P_i and [γ-^{32}P]ATP were used.

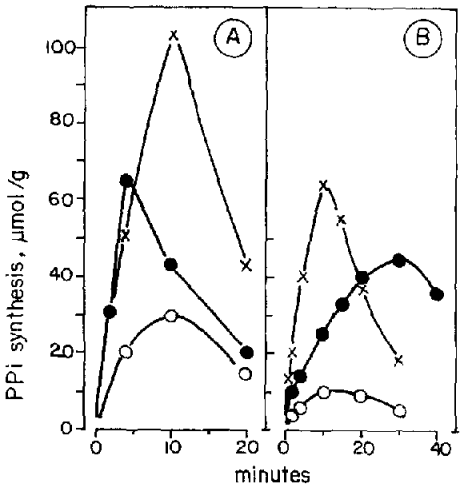

Fig.3. Pyrophosphate synthesis. $MgCl_2$ (A) and pH (B) dependence. In (A) the composition of the assay medium was 40 mM Mes-KOH buffer (pH 6.0), 4 mM $^{32}P_i$, 4 mM ATP and either (○) without added $MgCl_2$, (●) 8 mM $MgCl_2$ or (×) 24 mM $MgCl_2$. In (B) the composition of the assay medium was 4 mM $^{32}P_i$, 4 mM ATP, 8 mM $MgCl_2$ and 40 mM of the following buffers: (○) glycyl-glycine/KOH, pH 8.5; (●) Mops-KOH, pH 7.0; (×) Mes-KOH, pH 6.0. The reaction was started by the addition of enzyme to a final concentration of 40 μg/ml and arrested after different incubation intervals at 35°C by the addition of trichloroacetic acid to a final concentration of 10% (w/v).

traction to the organic phase as phosphomolybdate, the amount of radioactive material remaining in the aqueous phase was much less than that detected in the control experiments without inorganic pyrophosphatase (fig.2). This indicates that the pyrophosphate synthesized by alkaline phosphatase was simultaneously hydrolyzed by the pyrophosphatase allowing very little of it to accumulate in the medium. In a second set of experiments, the radioactive material formed during hydrolysis of either glucose 6-phosphate, glucose 6-phosphate plus ADP or ATP, under conditions described for figs 1A and 2, was isolated and characterized on thin-layer chromatograms (see section 2). This material had the same R_f as pyrophosphate, and after incubation for 5 min with inorganic pyrophosphatase the radioactive material moved at the front, alongside the control $^{32}P_i$.

The rate of pyrophosphate synthesis was faster in the presence of $MgCl_2$ (fig.3A) and increased as the pH of the medium was decreased from 8.5 to 6.0 (fig.3B). This was observed using either ATP (fig.3) or glucose 6-phosphate as substrate (data not shown).

Synthesis of radioactive pyrophosphate from $^{32}P_i$ was also detected during hydrolysis of *p*-nitrophenylphosphate, and of non-radioactive pyrophosphate (data not shown).

4. DISCUSSION

The differences between the velocities of either ATP or glucose 6-phosphate hydrolysis and that of pyrophosphate synthesis are related to kinetic parameters and not to the energies of hydrolysis of either the phosphoserine residue or the pyrophosphate molecule at the catalytic site of the enzyme. The rates of hydrolysis and synthesis depend on the unidirectional forward rate constants of reactions 3, 4 and 5 and not on the ratio between the forward and backward rate constants, which defines the equilibrium constant of the reaction. This has been discussed in detail in previous reports in connection with other enzymes [18,19].

Regardless of the substrate used by alkaline phosphatase, the first step of the catalytic cycle is the phosphorylation of a serine residue located at the catalytic site of the enzyme. Under the condi-

tions of our experiments this phosphate is then transferred either to a water molecule (reactions 3 and 4) or to another P_i molecule (reaction 5) leading to the synthesis of pyrophosphate. According to the ΔG° values previously determined in water and in organic solvents [5–8], this reaction would not be thermodynamically feasible if the activity of water were the same at the catalytic site as in the solution that surrounds the enzyme. Our finding indicates that at the catalytic site of this enzyme the ΔG° values for phosphoserine and pyrophosphate hydrolysis are similar to those measured in the presence of organic solvents [5].

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