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ENZYMIC DEPHOSPHORYLATION OF ADENOSINE DIPHOSPHATE PHOSPHOGLYCERIC ACID

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SUMMARY

An enzyme from muscle which acts on adenosine diphosphate phosphoglyceric acid and leads to the hydrolysis of the phosphate group at the 2-position of the glyceric acid moiety has been studied. The enzyme is strongly stimulated by inorganic pyrophosphate. This action is very specific. The enzyme also acts on 2,3-diphosphoglycerate.

INTRODUCTION

HASHIMOTO *et al.*[1–3] isolated from the blood of mammals a compound in which the phosphate in position 3 of 2,3-diphosphoglyceric acid is joined to the 5'-phosphate of AMP, forming a pyrophosphate bridge. HASHIMOTO and coworkers called the substance: adenylyl 2,3-diphosphoglyceric acid. It can also be called adenosine diphosphate (ADP) phosphoglyceric acid following the terminology used for nucleoside diphosphate sugars.

In the course of investigations on the action of enzymes on ADP-phosphoglyceric acid, it has been found that muscle extracts, in the presence of PP_i catalyze the removal of phosphate from the nucleotide according to the following equation:

$$\text{ADP-phosphoglyceric acid} + H_2O \longrightarrow P_i + \text{ADP-glyceric acid}$$

The enzyme has been found to act also on 2,3-diphosphoglyceric acid.

METHODS

Preparation of materials

ADP-glyceric acid and ADP-phosphoglyceric acid were synthesized from 3-phosphoglyceric acid or 2,3-diphosphoglyceric acid respectively and AMP morpholidate by the method of KHORANA and collaborators[4] with slight modifications[5]. Purification was carried out by anion-exchange chromatography with a LiCl gradient[4].

2,3-Diphosphoglycerate was obtained from human blood[6]. Cyclic 2,3-monophosphoglyceric acid was prepared by mixing the tributylamine salt of 3-phospho-

glyceric acid and dicyclohexylcarbodiimide in a 1:10 molar ratio in anhydrous pyridine. After 15 h at room temperature, the product was isolated by paper chromatography with isopropanol–ammonia as solvent[7].

Preparation of the enzyme

The skeletal muscles from a rabbit anesthesized with $MgSO_4$ were ground and extracted with 2 volumes of ice-cold water. The extract was passed through cheesecloth and stored frozen. Portions of 10 ml were mixed with 30 ml of methanol (at −20°). The mixture was centrifuged at 10 000 × *g* for 10 min and the precipitate was discarded. 30 ml methanol were again added to the supernatant fluid. The precipitate obtained by centrifugation was suspended in 1 ml of distilled water and dialyzed for 30 min against 1 mM mercaptoethanol. The extract was clarified by centrifugation and dialyzed for 1 h against pure glycerol. All operations were carried out at 0°. The procedure increased the specific activity 8- to 20-fold.

The preparations of 2,3-diphosphoglyceric acid phosphatase (EC 3.1.3.13) were obtained from chicken muscle as described by JOYCE AND GRISOLIA[8] or from rabbit muscle according to RAPOPORT AND LUEBERING[9]. Enolase (EC 4.2.1.11) was purified as described by BUCHER[10] as far as the second precipitation with ethanol.

Estimation of the enzyme

Several procedures were used.

Procedure a: Liberation of P_i: Trichloroacetic acid (10%) was added to the reaction mixture and after centrifugation the supernatant fluid was analyzed for P_i as described by KUNITZ[11] in a final volume of 0.4 ml. The usual procedures for P_i could not be used due to the presence of pyrophosphate which is hydrolyzed by the reagents.

Procedure b: The reaction product was hydrolyzed to phosphoglyceric acid, the latter was then converted enzymically to pyruvate which was estimated with DNP-hydrazine. The procedure was as follows. The reaction mixture (0.04 ml) was acidified with 0.02 ml of 2 N HCl, and the samples were heated for 10 min at 100°. The acid was neutralized with the exact amount of $KHCO_3$. The following substances were then added: 1 μmole of potassium phosphate (pH 7.3); 0.3 μmole of $MgSO_4$; 0.06 μmole of ADP, and 0.03 ml of crude rabbit-muscle extract. The samples were incubated for 15 min at 37°, the reaction was stopped by adding DNP-hydrazine solution and pyruvate was estimated as described previously[12]. A 3-phosphoglyceric acid standard and a blank were run at the same time. The absorbancy was proportional to 3-phosphoglyceric acid up to 0.12 μmole.

Procedure c: Paper chromatography was used for qualitative experiments. The samples were deproteinized by adding methanol to 75% concentration (in some cases enzyme action was not completely stopped under these conditions). After centrifugation the supernatant fluid was concentrated and the samples were spotted on Whatman No. 1 paper previously dipped in 0.01 M EDTA and dried (this avoids tailing). The solvent used for chromatography was ethanol–ammonium acetate (pH 3.8) (ref. 13). The mobility of the different compounds is shown in Table I.

RESULTS

The action of the enzyme on ADP-phosphoglyceric acid

Incubation of ADP-phosphoglyceric acid with the enzyme was found to lead to the formation of ADP-glyceric acid. This change could be detected by the fact that the reaction product runs faster during paper chromatography and slightly more slowly during electrophoresis in sodium carbonate–sodium bicarbonate buffer (pH 9.2) than ADP-phosphoglyceric acid (Table I). Another difference became apparent after heating the reaction product at pH 9–10 for 10 min at 100°. Under these conditions

TABLE I

MOBILITY OF SUBSTRATES, PRODUCTS AND REFERENCE COMPOUNDS

The reaction product heated at pH 9–10 for 10 min at 100° gave two products, A and B. Only A was ultraviolet absorbing. Acid hydrolysis of B was carried out with 1 N acid for 10 min at 100°. The phosphate-containing compounds were detected with BURROWS *et al.*'s[14] reagent. Paper electrophoresis was carried out at 14 V/cm for 1.5 h (ref. 15) in 0.25 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.2). Solvent for paper chromatography was ethanol–ammonium acetate (pH 3.8) (ref. 13)

Substance	*Paper electrophoresis* (R_{p_i})	*Paper chromatography* (R_{p_i})
Reaction product	0.61	0.53
Alkaline degradation Product A	0.52	0.82
Alkaline degradation Product B	1.13	1.0
B after acid hydrolysis	1.0	—
ADP-phosphoglyceric acid	0.70	0.48
ADP-glyceric acid	0.60	0.52
AMP	0.52	0.82
Cyclic 2,3-monophosphoglyceric acid	1.13	1.0
3-Phosphoglyceric acid	1.08	0.98
Phosphoglycollic acid	1.08	1.15
Phosphoenol pyruvic acid	1.05	0.98
2,3-Diphosphoglyceric acid	0.91	0.79
Dihydroxyacetonephosphate	0.84	—
Glycerolphosphate	0.84	1.15
PP_i	0.85	0.82

ADP-phosphoglyceric acid was not modified while the reaction product decomposed into two substances: one which behaved like AMP (A in Table I) and another, B, which contained phosphate and ran faster than 3-phosphoglyceric acid during electrophoresis. Substance B behaved like 3-phosphoglyceric acid after acid hydrolysis. Substance B was compared with a sample of cyclic 2,3-monophosphoglyceric acid and the two compounds were undistinguishable by electrophoresis or chromatography as shown in Table I. They also showed the same mobility when chromatographed with isopropanol–ammonia–water (70:10:20, v/v)[7]. Synthetic ADP-glyceric acid was found to be decomposed by alkali like the reaction product.

The action of PP_i

The addition of PP_i was found to produce a striking activation of the dephosphorylation of ADP-phosphoglyceric acid. In the absence of PP_i the reaction does

take place but can hardly be detected. A curve showing the action of PP_i is shown in Fig. 1. A LINEWEAVER–BURK[16] plot of these values gave $K_m = 20$ mM. The dephosphorylation of 2,3-diphosphoglyceric acid was also found to be activated by PP_i. In this case the K_m was 27 mM.

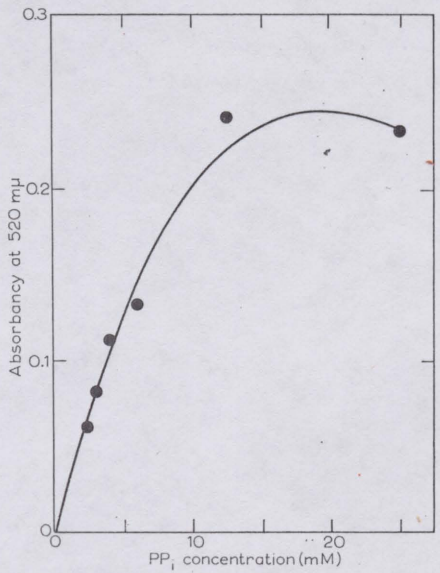

Fig. 1. Effect of PP_i concentration on enzyme activity. The reaction mixture contained: 0.2 μmole of $MgSO_4$; 0.076 μmole of ADP-phosphoglyceric acid; 0.02 ml of enzyme and the concentration of PP_i (pH 7.2) as indicated. Total volume was 0.06 ml. Incubation was carried out for 15 min at 37°. The enzyme was estimated as described in text (Procedure b).

Many substances were tested as possible substitutes for PP_i. The following compounds gave negative results: P_i or inorganic triphosphate, citrate, malate, oxalate, EDTA, glucose 1-phosphate, glucose 6-phosphate, AMP, ADP, ATP, UDP, UTP, arsenate, Hg^{2+} and Ag^{+}. The latter cations have been found to stimulate 2,3-diphosphoglycerate phosphatase[9].

On the other hand PP_i was tested as possible activator in the dephosphorylation of other compounds. None of the following compounds were found to be dephosphorylated by the enzyme either with or without PP_i: glucose 1-phosphate, fructose 1,6-diphosphate, AMP, ATP, α- or β-glycerophosphate.

Activity under various conditions

As shown in Fig. 2 maximal activity was obtained at pH 6.6. Mg^{2+} was found to be without effect. The rate of reaction was linear with time up to 60 min.

The comparison of different enzyme preparations

The enzyme preparation was found to act not only on ADP phosphoglyceric acid but also on 2,3-diphosphoglycerate and since specific phosphatases for the latter compound have been studied, it was considered of interest to compare the

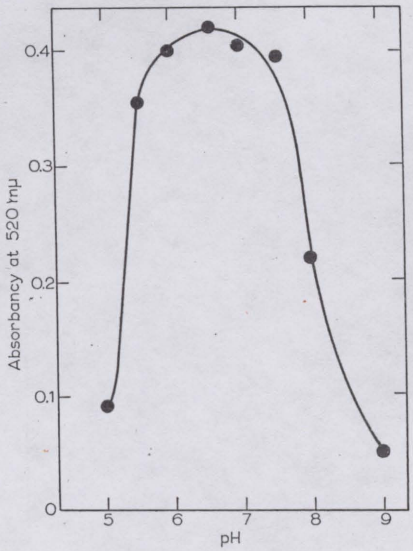

Fig. 2. pH–activity curve. The conditions were as in the assay of Fig. 1 but with 1 μmole of PP_i buffer.

action of different preparations. The procedure described by RAPOPORT AND LUEBERING[9] starts with rabbit muscle, includes a precipitation with ethanol and is not very different from that described in this paper. The chicken-muscle preparation of JOYCE AND GRISOLIA[8] includes treatment of the enzyme with $HgCl_2$.

It may be observed in Table II that the JOYCE AND GRISOLIA preparation acts very slowly on ADP-phosphoglyceric acid and is not influenced by PP_i. The enzyme prepared according to RAPOPORT AND LUEBERING is similar to that described in this paper. Both act slightly more on ADP-phosphoglyceric acid than on 2,3-diphosphoglyceric acid and show similar activation by PP_i.

TABLE II

THE ACTION OF DIFFERENT ENZYME PREPARATIONS

The reaction mixture contained: 0.3 μmole of 2,3-diphosphoglyceric acid or 0.27 μmole of ADP-phosphoglyceric acid, 1 μmole of PP_i (pH 7.2) or 1 μmole of Tris (pH 7.2) and 0.3–0.1 mg of enzyme. Final volume 0.04 ml. After 30 min at 37° the reaction was stopped with 10% trichloroacetic acid. P_i was estimated as described in text. Results are expressed in μmoles of P_i per mg protein per 30 min.

Substrate	*Addition*	*Enzyme preparation*		
		Joyce and Grisolia	*Rapoport and Luebering*	*This paper*
2,3-Diphosphoglyceric acid	None*	2.6	0	0
2,3-Diphosphoglyceric acid	PP_i	2.4	2.8	5.0
ADP-phosphoglyceric acid	None*	0.41	0	0.06
ADP-phosphoglyceric acid	PP_i	0.63	4.4	6.2
None	PP_i	0.09	0.05	0.015

* PP_i was added after incubation.

The position of the phosphate in the reaction product

In order to ascertain which hydroxyl of glyceric acid is joined to the ADP moiety in the reaction product, the latter was hydrolyzed with a nucleotide pyrophosphatase (EC 3.6.1.9) and then the phosphoglyceric acid formed was tested with enolase, which is known to act only on 2-phosphoglyceric acid. The experiment was carried out as follows: 6 μmoles of ADP-phosphoglyceric acid and 4 μmoles of PP_i of pH 7.2 were incubated with enzyme for 1 h at 37°, and then heated 1 min at 100°. Paper chromatography of an aliquot with ethanol–ammonium

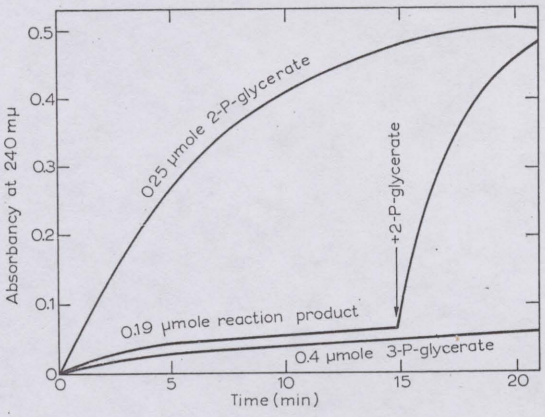

Fig. 3. Action of enolase on the reaction product treated with pyrophosphatase. The treatment with pyrophosphatase is described in text. An aliquot (containing 0.19 μmole of phosphoglyceric acid) was added to the components of the enolase test[10]. At the point indicated, 2-phosphoglyceric acid was added.

acetate as solvent showed that about half the substrate had been transformed. The rest of the incubation mixture was mixed with 0.5 M glycine buffer (pH 8.6), Mg^{2+} to a final concentration of 0.05 M and 4 μg of *Crotalus adamanteus* venom. After 20 min at 37° the enzyme was inactivated by heating and the nucleotides were adsorbed on charcoal (50 mg of Norit A). The filtrate was analyzed for phosphoglyceric acid as described under "Estimation of the enzyme" (Procedure b) and found to contain 1.6 μmoles. This solution was then tested with enolase as described by BUCHER[10]. As shown in Fig. 3 the phosphoglyceric acid in the solution behaved like 3-phosphoglyceric acid, that is, it was not acted upon by enolase. Addition of 2-phosphoglyceric acid showed that the lack of action was not due to inhibiting substances.

DISCUSSION

The evidence shows that the reaction catalyzed by the enzyme consists in a removal of a phosphate group from ADP-phosphoglyceric acid. A free hydroxyl is left vicinal to the one which carries the pyrophosphate group. The compound then becomes labile to alkali and decomposes into AMP and cyclic 2,3-monophosphoglyceric acid. The sensitivity to alkali is similar to that of other nucleoside diphosphate sugars such

as UDP-glucose[13] which have a free hydroxyl in position suitable for the formation of a 5-membered cyclic phosphate.

The reaction product has the pyrophosphate-bond joined at position 3 of the glyceric acid. Thus by the action of a pyrophosphatase it gives rise to 3-phosphoglyceric acid. These facts indicate that the enzyme removes the phosphate at position 2 of ADP-phosphoglyceric acid.

As to the mechanism by which PP_i activates the reaction, no positive evidence was obtained. Preliminary experiments with [^{32}P]PP_i or ADP-[^{32}P]phosphoglyceric acid followed by paper chromatography indicated that the reaction consisted in a direct removal of the phosphate from ADP-phosphoglyceric acid and that the pyrophosphate remained as such. Therefore PP_i seems to act directly by modifying the activity of the enzyme. Whether this action, which is very specific, is part of a regulatory mechanism is a question which will have to be investigated.

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