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PHOSPHATE TRANSFER FROM ADENOSINE DIPHOSPHATE PHOSPHOGLYCERATE AND 2,3-DIPHOSPHOGLYCERATE

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SUMMARY

The following phosphate transfer reaction has been found to be catalyzed by muscle and yeast extracts:

2,3-Diphosphoglycerate could also be used as donor and 3-phosphoglycerate as acceptor. The activity was found to be parallel to that of adenosine diphosphate phosphoglycerate phosphatase during purification and in enzyme preparations of different origin. Some properties of the enzyme have been studied.

INTRODUCTION

The metabolic role of ADP-phosphoglycerate (adenylyl 2,3-diphosphoglycerate) is not known. This compound was isolated from mammalian blood by HASHIMOTO¹ and has been found² to be hydrolyzed by a specific phosphatase to give ADP-glycerate. This phosphatase, which also hydrolyzes 2,3-diphosphoglycerate, was found to be strongly activated by inorganic pyrophosphate. Further work has been carried out, designed to obtain information on the metabolism of ADP-phosphoglycerate. After incubation of the [¹⁴C]-labeled compound with yeast or muscle extracts some transfer reactions were detected, which turned out to be catalyzed by the same enzyme described in the previous paper². The reactions are as follows:

In reaction (1) transfer of phosphate takes place from position 2 of the phosphoglycerate residue of one compound to the other. A somewhat similar reaction is

catalyzed by phosphoglyceric acid mutase (D-2,3-diphosphoglycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3.) as follows:

In this case transfer takes place reversibly from positions 2 to 3. This paper reports some studies on the transfer reactions.

EXPERIMENTAL

Materials

ADP-phosphoglycerate and ADP-glycerate were prepared as previously described². The preparation of 3-[¹⁴C]phosphoglycerate was carried out as described by JACOBY *et al.*³ and 2,3-diphosphoglycerate was obtained from it as described by GRISOLIA⁴. Both compounds were purified by paper chromatography with solvent C₁.

Analytical methods

Protein was measured according to LOWRY *et al.*⁵. Phosphoglycerate mutase was assayed according to RODWELL *et al.*⁶ by the enolase coupled method⁷. Phosphatase was determined under the conditions previously described² but phosphate was estimated as described below.

Paper chromatography was carried out with the following solvents: C₁, ethanol-ammonium acetate, pH 3.8 (ref. 8); C₂, ethanol-ammonium acetate, pH 7.5 (ref. 8).

The solvents used for paper electrophoresis were: E₁, pyridine acetate, pH 6.5 (pyridine-acetic acid-water, 10:0.4:90, v/v), 22 V·cm⁻¹ for 75 min (ref. 9); E₂, 0.2 M ammonium formate, pH 3.6, 13 V·cm⁻¹ for 1 h (ref. 10).

Estimation of phosphate in the presence of pyrophosphate

In many of the phosphatase assays the method of KUNITZ¹¹ was used for the estimation of P_i. However, since in this method interference by PP_i is not completely avoided, another procedure was developed. This consists in the removal of PP_i as the manganese salt¹² before measuring phosphate according to FISKE AND SUBBAROW¹³. The method was as follows: the reaction was stopped by heating for 2 min at 100°. After cooling, water was added to complete 0.1 ml, followed by 0.04 ml of 1 M acetate buffer, pH 5, and 0.04 ml of 0.1 M MnCl₂. After 15 min at room temperature, the mixture was centrifuged. An aliquot of the supernatant fluid was transferred to another tube. Usually 0.1 ml was taken with a Lang-Levy pipette. The P_i was then estimated by the method of FISKE AND SUBBAROW¹³ in a final volume of 0.5 ml. Part of the P_i (about 25%) was carried down with the manganese pyrophosphate but this could be corrected by appropriate standards. The absorbance was proportional to the amount of P_i up to 0.1 μmole.

Enzymes

Purified chicken muscle and yeast phosphoglycerate mutase were generously provided by Dr. Santiago Grisolia. Rabbit muscle phosphatase was prepared as previously described². The precipitate obtained between 3 and 4 vol. of methanol was dissolved in water and dialyzed for 30 min against 5 mM mercaptoethanol. The

enzyme preparation (about 84 mg of protein) was then applied to a DEAE-cellulose column (2×25 cm) equilibrated with 5 mM Tris buffer (pH 8.2) containing 5 mM mercaptoethanol. The latter solution was used as eluent. The phosphatase and transferase activity appeared in the percolate and first washings. The active fractions were pooled and the enzyme was concentrated by dialysis against glycerol. The DEAE-cellulose-treated enzyme was free from inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1).

Measurement of the transfer reaction

A method was developed based on the fact that ADP-glycerate, but not ADP-phosphoglycerate, is decomposed by mild alkali². The procedure was as follows: the reaction mixture contained (in μ moles): 0.075 of ADP-phosphoglycerate, 0.027 of

Fig. 1. Transfer reaction with different amounts of enzyme. Procedure as described in text.

ADP- ^{14}C glycerate (7500 counts/min), 2.5 of mercaptoethanol, 5 of Tris buffer of pH 7.2, and enzyme. Final volume 0.05 ml. After 30 min at 37° , the mixture was made alkaline to thymol blue (as internal indicator) by adding about 0.04 ml of 1 N NaOH. The mixture was heated to 100° for 10 min and after cooling, 0.07 ml of 25% perchloric acid and 0.45 ml of water were added. The precipitate was centrifuged off and charcoal (about 9 mg of Norit A) was added to the supernatant fluid. The suspension was shaken for 10 min and then centrifuged. The precipitate was washed three times with 1 ml of 0.1 M K_2HPO_4 , plated on aluminium planchets and dried. The radioactivity was measured with a gas-flow counter. Self-absorption of these samples amounted to about 70%, but no correction was applied for it.

Fig. 2. Time course of the transfer reaction. Procedure as described in text.

Fig. 3. Effect of donor concentration on the transfer reaction. The reaction mixture and assay were as described in text. ADP-phosphoglycerate and 2,3-diphosphoglycerate in the indicated concentrations.

The method was tested with different amounts of enzyme and, as shown in Fig. 1, the number of counts was found to be proportional to enzyme concentration, within certain limits. A time curve is shown in Fig. 2. The effect of varying the concentration of donor is shown in Fig. 3.

RESULTS

Detection of transfer by paper chromatography

Incubation of labeled ADP-glycerate with ADP-phosphoglycerate and rabbit muscle phosphatase led to the formation of a radioactive compound which migrated like ADP-phosphoglycerate on paper chromatography with solvent C₁. An experiment of this type in which the radioactivity of different zones of the paper was measured is shown in Table I. It may be observed that 2,3-diphosphoglycerate could be sub-

TABLE I

PHOSPHATE TRANSFER REACTIONS

The reaction mixture contained: 5 μ moles of Tris buffer of pH 7.2; 2.5 μ moles of mercaptoethanol; 0.075 μ moles of ADP-phosphoglycerate or 2,3-diphosphoglycerate; 0.02 ml of DEAE-cellulose-treated enzyme; 0.054 μ moles of ADP-glycerate (15 000 counts/min) or 0.067 μ moles of 3-[¹⁴C]-phosphoglycerate (10 000 counts/min). Final volume: 0.055 ml. Incubations were carried out at 37° for 30 min. The reaction was stopped with 5% trichloroacetic acid (final concentration), the tubes were kept in the cold in order to avoid decomposition of ADP-glycerate. Trichloroacetic acid was removed by washing three times with ether. The reaction mixture was adjusted to pH 7 with dilute NH₃. The samples were concentrated and spotted on Whatman No. 1 paper previously dipped in 0.01 M EDTA². After chromatography with solvent C₁, the papers were cut into 1-cm portions and counted in a thin-window gas-flow counter.

¹⁴ C-Acceptor	P-donor	Counts/min in ADP- glycerate zone	Counts/min in ADP- phosphogly- cerate zone	Per cent transferred
ADP-glycerate	—	1254	0	0
ADP-glycerate	ADP-phosphoglycerate	862	373	30
ADP-glycerate	2,3-diphosphoglycerate	1058	225	18

¹⁴ C-Acceptor	P donor	Counts/min in 3-phos- phoglycerate zone	Counts/min in 2,3-di- phosphogly- cerate zone	Per cent transferred
3-Phosphoglycerate	—	524	0	0
3-Phosphoglycerate	ADP-phosphoglycerate	318	199	39
3-Phosphoglycerate	2,3-diphosphoglycerate	311	241	44

stituted for ADP-phosphoglycerate as phosphate donor. When labeled 3-phosphoglycerate was incubated with ADP-phosphoglycerate or 2,3-diphosphoglycerate, radioactivity appeared in the 2,3-diphosphoglycerate zone of the paper.

Experiments with the yeast enzyme showed that it behaved like the muscle enzyme. Thus, on adding ADP-phosphoglycerate, ADP-[¹⁴C]glycerate and enzyme, a radioactive product was formed which migrated like ADP-phosphoglycerate on paper chromatography.

Identification of the reaction product

The charcoal from several planchets which had been used for measuring the transfer reaction was collected in a centrifuge tube, washed several times with water and eluted with 0.15 N ammonia in 40% ethanol. The radioactive product was identified by paper chromatography and electrophoresis. It was then hydrolyzed (1 N HCl at 100° for 15 min)¹ and run again. The results appear in Table II where it

TABLE II

IDENTIFICATION OF THE REACTION PRODUCT

Compound	Solvents			
	Paper chromatography		Paper electrophoresis	
	C ₁	C ₂	E ₁	E ₂
Reaction product	0.21	0.15	0.83	0.83
ADP-phosphoglycerate	0.22	0.15	0.86	0.86
ADP-glycerate	0.35	0.43	0.78	0.76
2,3-Diphosphoglycerate	0.43	0.15	1.21	1.17
3-Phosphoglycerate	0.89	0.74	1.17	1.11
Reaction product after hydrolysis	0.55	0.20	1.22	1.20
2,3-Diphosphoglycerate	0.56	0.17	1.24	1.22
ADP-phosphoglycerate	0.23	0.17	0.94	0.87
ADP-glycerate	0.44	0.37	0.74	0.75
3-Phosphoglycerate	0.89	0.76	1.15	1.17

Data are expressed as R_F .

may be observed that the reaction product behaves like ADP-phosphoglycerate and that after hydrolysis it runs on paper like 2,3-diphosphoglycerate.

Transfer from ADP-phosphoglycerate treated with muscle phosphatase

The chemical synthesis of ADP-phosphoglycerate is carried out by reaction of AMP morpholidate with 2,3-diphosphoglycerate¹⁴. Theoretically two isomers could be formed, depending on whether the AMP residue condenses with the 2 or the 3 phosphate group of 2,3-diphosphoglycerate. These will be called the 2 or the 3 isomer respectively. HASHIMOTO AND YOSHIKAWA¹⁵ found that AMP morpholidate reacts better with 3-phosphoglycerate (45% yield) than with 2-phosphoglycerate (10% yield) and concluded that the compound formed from 2,3-diphosphoglycerate must be the 3 isomer. It was previously reported² that the muscle phosphatase acting on ADP-phosphoglycerate gave ADP-glycerate with the ADP residue joined to position 3 of glycerate. This proved that the enzyme removes the phosphate from position 2 of the 3 isomer but did not exclude the presence of some 2 isomer in the substrate preparation.

In the study of the transfer reaction it was important to decide whether transfer takes place from the 2 or the 3 isomer of ADP-phosphoglycerate. Therefore experiments were carried out in which ADP-phosphoglycerate preparations were first treated

TABLE III

TRANSFER FROM ADP-PHOSPHOGLYCERATE PREPARATION AFTER THE ACTION OF PHOSPHATASE

Expt. I: Treatment with purified rabbit muscle phosphatase was carried out by incubating at 37° the following mixture: 0.25 μ mole of ADP-phosphoglycerate, 7.5 μ moles of mercaptoethanol, 3 μ moles of PP_i and 0.06 ml of enzyme. Total volume 0.2 ml. After 1 h, more enzyme (0.06 ml) was added. After another hour the reaction was stopped with trichloroacetic acid. A control in which the enzyme was added after incubation was run at the same time. The samples were chromatographed on paper with solvent C₁ and the ADP-phosphoglycerate zone was eluted with water. A half of each sample was tested as donor for transfer with the DEAE-cellulose-treated rabbit muscle enzyme and ADP-[¹⁴C]glycerate by the charcoal method as described in text. Expt. II was carried out by a similar procedure.

Phosphate donor	Counts/min in charcoal	
	Expt. I	Expt. II
None	42	88
ADP-phosphoglycerate	266	630
Phosphatase treated ADP-phosphoglycerate	58	63

exhaustively with muscle phosphatase and then tested as donors in the transfer reaction. As shown in Table III, phosphatase-treated ADP-phosphoglycerate was inactive as phosphate donor. Thus it may be concluded that either the 2 isomer is not used as donor or that it is not present in the ADP-phosphoglycerate preparations.

Assay of enzymes in different fractions

The three activities: phosphatase, mutase and transferase were tested in the different fractions obtained in the purification of the rabbit muscle enzyme. The results are shown in Table IV. It was found that the specific activity of the three enzymes increased, but that of the mutase less than the others.

Many experiments were carried out in order to separate the three activities. DEAE-cellulose, DEAE-Sephadex, carboxymethyl-cellulose, Sephadex G-100, Sephadex G-200 were used without success.

In order to find out whether the three activities corresponded to different enzymes, different purified preparations were compared. The chicken breast muscle

TABLE IV

PURIFICATION OF THE RABBIT MUSCLE ENZYME

Procedures were as described in the text.

Fraction	Volume (ml)	Protein (mg/ml)	Transferase (counts/min per mg per min)	Phosphatase (μ moles/mg per min)	Mutase (units/mg)
Crude	340	24	64	7.3	77
Methanol precipitate	35	2.4	500	113	215
After DEAE-cellulose treatment	13	0.74	2750	423	545
Purification factor			43	59	7.1

TABLE V

ACTIVITY OF ENZYMES OF DIFFERENT ORIGIN

Activities measured as described in the text. Phosphatase was assayed with 0.09 μ mole of 2,3-diphosphoglycerate as substrate, by the method in which PP_i is precipitated with Mn^{2+} .

	Mutase* (units/mg)	Transferase (counts/ min per mg per min)	Phosphatase (μ moles/ mg per min)
Chicken breast muscle phosphoglycerate mutase	560	2420	70
Yeast phosphoglycerate mutase	590	2410	83
Rabbit muscle phosphatase after DEAE-cellulose treatment	360	2550	73

* Dialyzed for 2 h against water and 1 h against glycerol.

phosphoglycerate mutase preparation was found to yield P_i on incubation without added substrate, but after dialysis the phosphatase activity could be determined. As shown in Table V, the three enzyme preparations were found to have very similar specific activities. This was a very curious finding considering that the preparations were from different organisms and that the methods of purification were different. Some of the measurements were checked several times.

Specificity of the transfer reaction

As mentioned before, it was found by paper chromatography that both ADP-phosphoglycerate and 2,3-diphosphoglycerate could serve as donors for ADP-glycerate and that 3-phosphoglycerate could serve also as acceptor.

In most experiments with the rabbit muscle phosphatase preparation, more transfer was obtained with ADP-phosphoglycerate than with 2,3-diphosphoglycerate as donor. Using the charcoal method many other substances were tested as possible donors. Negative results were obtained with: fructose-1,6- P_2 , UTP, GTP, ITP, CDP, ATP, glucose-1- P , glucose-1,6- P_2 , phosphoenol pyruvic acid, fructose-1- P , glucose-6- P , PP_i , 2-phosphoglycerate, 3-phosphoglycerate.

The activation of phosphoglycerate mutase

ADP-phosphoglycerate and 2,3-diphosphoglycerate were compared as substrates

TABLE VI

COMPARISON BETWEEN 2,3-DIPHOSPHOGLYCERATE AND ADP-PHOSPHOGLYCERATE IN DIFFERENT REACTIONS

Methods as described in text.

Addition	Phosphatase (μ moles P_i /min)	Mutase (Δ absorbance/min)	Transferase (counts/min per mg per min)
2,3-Diphosphoglycerate	68	0.20	517
ADP-phosphoglycerate	80	0.07	1747
None	—	0.02	—

TABLE VII

EFFECT OF VARIOUS SUBSTANCES ON THE TRANSFER REACTIONS

Reaction mixture as described in text. Assay by the charcoal method.

<i>Substance added</i>	<i>Concentration (mM)</i>	<i>Relative activity (%)</i>
None	—	100
3-Phosphoglycerate	1.6	9
2-Phosphoglycerate	1.6	6
2,3-Diphosphoglycerate	1.6	30
None	—	100
MgCl ₂	18	32
MnCl ₂	1	19
NaCl	18	112
PP _i	9	0
P _i	9	70
EDTA	18	72
EDTA	2	113

Fig. 4. Heat stability of the enzymes. The activities were measured as described in text after the enzymes were heated for 5 min at the indicated temperatures. The activity before heating was taken as 100%. The enzyme preparation was rabbit muscle phosphatase after DEAE.

for the phosphatase and transferase and as activators of the mutase. As shown in Table VI, both compounds are hydrolyzed by the rabbit phosphatase preparation in the presence of PP_i . They also serve as donors in the transfer to ADP-glycerate. In this reaction, ADP-phosphoglycerate gave a higher yield than 2,3-diphosphoglycerate. When the two compounds were tested as activators of the mutase, it was found that ADP-phosphoglycerate was less active than 2,3-diphosphoglycerate. With the enzyme preparation used in our experiments, it is likely that ADP-phosphoglycerate transferred phosphate to 3-phosphoglycerate giving 2,3-diphosphoglycerate. Thus the activation of the mutase would be indirect.

The effect of various substances on the transfer reaction

The effect of various substances on the transfer reaction is shown in Table VII. Phosphoglycerate inhibits presumably by competing for ADP-glycerate as acceptor. Magnesium and manganese chloride acted as inhibitors. PP_i was found to be a strong inhibitor at concentrations which activate the phosphatase. Since the transfer goes first through a maximum (Fig. 2) and then decreases, it was considered advisable to try the action of PP_i under several conditions. It was therefore tested with lower amounts of enzyme and for shorter periods of incubation and in every case it was found to inhibit transfer.

Heat stability

The effect of heating the different enzyme preparations is shown in Fig. 4. It may be observed that the inactivation of the phosphatase and transferase was parallel whereas the mutase was somewhat more resistant at 40–60°.

DISCUSSION

The activity of the phosphatase and of the transferase was parallel in different steps of purification of the rabbit muscle enzyme and in enzyme preparations of different origin. Thus it seems likely that both processes are catalyzed by the same enzyme.

The transfer of phosphate from ADP-phosphoglycerate to ADP- $[^{14}C]$ glycerate can be considered as an exchange reaction. It is similar to other cases which have been described such as the exchange of $[^{14}C]$ glucose with glucose-6-*P* catalyzed by glucose 6-phosphatase¹⁶ and that of $[^{14}C]$ serine with serine phosphate catalyzed by serine phosphatase¹⁷. In these cases it is assumed that an enzyme-phosphate intermediate is formed. A similar mechanism can be written for the reaction described in this paper.

Phosphatase action would be the sum of 4 plus 5 and the transfer or exchange would be 4 plus 6. The action of PP_i , which accelerates phosphatase and inhibits transfer might be explained as an increase in the rate of reaction (5).

A problem which could not be decided clearly is whether phosphoglycerate mutase is a different enzyme from the other two. During the purification procedure

the purification factor for the mutase was about 7 times lower. The inactivation by heating was slightly greater for the phosphatase and transferase reactions.

Furthermore 2,3-diphosphoglycerate was a better activator of the mutase than ADP-phosphoglycerate¹⁸. These results indicate that hydrolysis and transfer of phosphate are catalyzed by the same enzyme, but that the mutase reaction is probably catalyzed by a different protein.

The transfer of phosphate from ADP-phosphoglycerate to ADP-glycerate may not be a physiological reaction; the normal substrate might be 2,3-diphosphoglycerate. However, the reaction with the nucleotides permits a differentiation between a 2→2 transfer from the 2→3 transfer which is catalyzed by phosphoglycerate mutase.

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