STUDIES ON CRYSTALLINE YEAST PHOSPHOGLUCOMUTASE: THE PRESENCE OF INTRINSIC ZINC

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SUMMARY

Yeast phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) was inhibited by EDTA and was active without any addition of a bivalent cation. After the enzyme was dialyzed against the EDTA solution, the enzyme activity was recovered by removal of EDTA by passage through a Sephadex column. These observations suggested that the yeast enzyme contained an intrinsic metal ion essential for catalytic activity and that the EDTA inhibition was due to the formation of the enzyme–metal–EDTA ternary complex.

The enzyme activity was protected from EDTA inhibition in the presence of the coenzyme. About 1 mole of zinc per mole of the enzyme was observed by atomic absorption measurements. These observations suggested that the intrinsic zinc might exist on the coenzyme binding site.

The existence of intrinsic zinc in yeast phosphoglucomutase is in contrast to the muscle enzyme which requires extrinsic Mg^{2+} for activity. The yeast enzyme catalyzes the reaction by a different pathway from the muscle enzyme. The role of bivalent cations in the phosphoglucomutase reaction mechanism is discussed.

INTRODUCTION

It has been reported that the reaction sequence of phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) from rabbit muscle[1-5], shark muscle[6], flounder muscle[6] and *Escherichia coli*[7,8] is of the "ping-pong" type. However, it has been shown by kinetic experiments that the reaction sequence of phosphoglucomutases from yeast[9], *Micrococcus lysodeikticus*[8] and *Bacillus cereus*[8] is "sequential". The reaction sequences of phosphoglucomutases from various origins are, therefore, not identical.

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

In the present work phosphoglucomutase from yeast has been studied in contrast to the well-known rabbit muscle enzyme, based on the idea that studies on phosphoglucomutase proteins would be useful for an understanding of the relationship between the enzyme reaction mechanism and protein structure. In the previous study[10], the results of the amino acid analysis and molecular weight determination of yeast phosphoglucomutase did not show any particular characteristics compared to the rabbit muscle enzyme. The yeast enzyme was different to the muscle enzyme with respect to the effect of bivalent cations on the catalytic activity. It was reported that rabbit muscle phosphoglucomutase required Mg^{2+} for catalytic activity and was stimulated by the simultaneous addition of a chelating agent and Mg^{2+} (see refs 11–14). This effect was elucidated by the removal of inhibitory metal attached to the enzyme and by the addition of essential Mg^{2+} (see refs 15 and 16). On the other hand, the yeast enzyme was active without any addition of bivalent cations to the reaction mixture, and was strongly inhibited by chelating agents[9]. The stimulation by the simultaneous addition of Mg^{2+} and chelating agents was also observed in the yeast enzyme reaction[9].

In this report, mechanisms of inhibition and stimulation by EDTA in the yeast phosphoglucomutase reaction are described.

MATERIALS AND METHODS

Reagents

Glucose 1-phosphate, glucose 6-phosphate and glucose 1,6-diphosphate were purchased from Boehringer, Mannheim. 1-Anilino-8-naphthalene sulfonate (ANS) and the standard metal ion solutions (1.0 mg/ml $ZnCl_2$ and 1.0 mg/ml $MgCl_2$) were obtained commercially.

Glass-distilled water, which was obtained by glass distillation of deionized water, was used in all experiments. Tris buffer (pH 7.5), citrate buffer (pH 5.3) and acetate buffer (pH 5.3) were purified by passage through Amberlite IR-120 columns, previously equilibrated with the respective buffer. Glucose 1-phosphate, glucose 6-phosphate and glucose 1,6-diphosphate were also purified using Amberlite IR-120 (H^+-form).

Crystalline yeast phosphoglucomutase was prepared as described in the previous paper[9]. The concentration of the enzyme was determined on the basis of the molecular extinction coefficient at 280 nm: $8.2 \cdot 10^7$ cm^2/mole (see ref. 10).

Assay of the enzyme activity

The reaction mixture contained 1.6 μmoles of glucose 1-phosphate, 0.01 μmole of glucose 1,6-diphosphate and 5 μmoles of Tris buffer (pH 7.5) in a final volume of 0.4 ml. After incubation at 25 °C for 10 min, the reaction was initiated by the addition of the enzyme solution. The reaction was terminated by the addition of 4.6 ml of 0.54 M H_2SO_4. Acid-labile phosphate was determined by the method of Fiske and SubbaRow[17].

Atomic absorption measurements

The metal content of the enzyme was determined with Nippon Jarrell-Ash atomic absorption spectrophotometer (Model AA-70) equipped with a Hitachi

Recorder (Model QPD 34). Absorption wavelengths were 213.86 nm and 285.21 nm for the measurement of zinc and magnesium, respectively. Standard metal solutions were used after dilution with the same buffer as the medium of the enzyme solution.

Fluorescence measurements

The fluorescence intensity of ANS bound by the enzyme was measured with a Hitachi fluorescence spectrophotometer (Model MPF-2A). The excitation and the emission wavelengths were 400 nm and 470 nm, respectively. The number of ANS molecules bound by the enzyme was determined as described in the previous paper[10].

RESULTS

Stimulatory and inhibitory effects of EDTA

When the reaction was started by the addition of the enzyme dissolved in 10 mM acetate buffer (pH 5.3), the enzyme activity was stimulated by low concentrations of EDTA and was inhibited by high concentrations of EDTA as shown in Fig. 1.
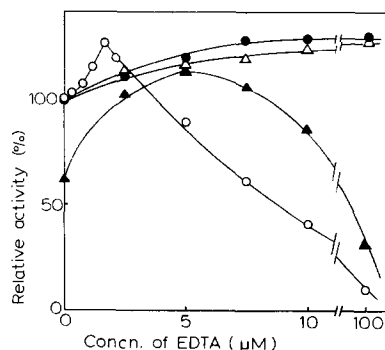

Fig. 1. Effects of EDTA and bivalent cations on the yeast phosphoglucomutase reaction. The reactions were started by the addition of the enzyme dissolved in 10 mM acetate buffer (pH 5.3). Addition to the reaction mixture: ○—○, none; ●—●, 1.0 mM Mg^{2+}; △—△, 0.50 mM Mn^{2+}; ▲—▲, 5.0 μM Zn^{2+}.

EDTA exerted two types of effect on the yeast enzyme activity without any addition of a bivalent cation. It is also shown in Fig. 1 that EDTA exerts only a stimulatory effect on the enzyme activity in the presence of Mg^{2+} or Mn^{2+}. A higher concentration of EDTA was required for the stimulation of the enzyme in the presence of Mg^{2+} and Mn^{2+} than in the absence of the bivalent cations, and the extent of the stimulation was identical under both conditions. These observations suggest that bivalent cations are not essential for the stimulation but they protect the enzyme activity from EDTA inhibition by decreasing the concentration of free EDTA. Thus, EDTA exerted a stimulatory effect in the presence of Zn^{2+}, which itself showed an inhibitory effect.

It was assumed from these observations that stimulation and inhibition by EDTA occurred at different sites on the enzyme surface. This was supported by other experimental results. When the reaction was started by addition of the enzyme dissolved in 10 mM Tris buffer (pH 7.5), EDTA exerted only a stimulatory effect, and the presence of Mg^{2+} reduced the effects of EDTA as shown in Fig. 2. Under these

conditions, the enzyme activity was completely protected from EDTA inhibition without any addition of a bivalent cation.

The enzyme activity was decreased to 3–70% of the native enzyme activity by preincubation of the enzyme with various bivalent cations (0.20 mM Co^{2+}, 20 μM Cu^{2+}, 20 μM Zn^{2+} and 2.0 μM Hg^{2+}) in Tris buffer (pH 7.5). The reduced enzyme activities were almost completely recovered by the addition of 0.1 mM EDTA to the reaction mixture with the exception of the Hg^{2+}-treated enzyme. This suggests that the stimulatory effect of EDTA is due to removal of inhibitory metal(s) attached to the enzyme.

The formation of the enzyme–EDTA complex

As shown in the previous paper[9], the extent of inhibition by chelating agents (citrate, cysteine and EDTA) is in the order of magnitude of stability constants between the agents and free metal ions. The yeast enzyme is active without any addition of a bivalent cation[9]. An essential bivalent cation, therefore, may be strongly bound by the yeast enzyme.

The yeast enzyme was dialyzed against 10 mM citrate buffer (pH 5.3) containing 0.1 mM EDTA which almost completely inactivates the enzyme (see Fig. 1). However, the activity was completely recovered on lowering the EDTA concentration by dilution of the enzyme solution or by the removal of EDTA by passage through a Sephadex G-50 column equilibrated with 10 mM Tris buffer (pH 7.5). These observations suggest that EDTA inhibition is not due to the removal of the essential metal ion from the enzyme, but to the formation of the enzyme–metal–EDTA complex.

The yeast enzyme activity was not inhibited by EDTA when the reaction was started by addition of the enzyme dissolved in Tris buffer (pH 7.5), as shown in Fig. 2. However, the activity was decreased by preincubation of the enzyme with EDTA in Tris buffer as shown in Fig. 3. The extent of the inhibition did not change even if
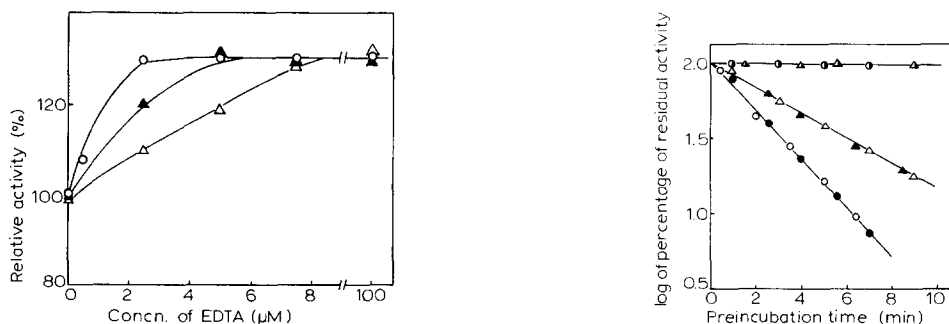

Fig. 2. Effects of EDTA and Mg^{2+} on the yeast phosphoglucomutase reaction. The reactions were started by the addition of the enzyme dissolved in 10 mM Tris buffer (pH 7.5). Addition of Mg^{2+} to the reaction mixture: ○—○, none; ▲—▲, 0.50 mM; △—△, 2.0 mM.

Fig. 3. The time-dependent inhibition of EDTA on the yeast phosphoglucomutase reaction. The enzyme (0.50 μM) was preincubated with EDTA in 10 mM Tris buffer (pH 7.5) at 25 °C. The reactions were started by the addtion of 40 μl of the enzyme to 0.36 ml of the mutase reaction mixture. The mutase reactions were carried out as described in the text. The ordinate represents the logarithm of the percentage of residual activity relative to the activity without the preincubation. Additions of EDTA to the preincubation mixture: 10 μM (○, ●, ◑), 5.0 μM (△, ▲, ◮). Additions of EDTA to the mutase reaction mixture: none (◑, ◮), 20 μM (●, ▲), 0.20 mM (○, △).

higher concentrations of EDTA than those in the preincubation mixture were added to the mutase reaction mixture. These observations suggest that the enzyme–EDTA complex is formed during the preincubation, and that EDTA in the mutase reaction prevents the dissociation of the enzyme–EDTA complex thus formed.

It is clear from Fig. 3 that formation of the enzyme–EDTA complex follows apparent first-order kinetics. This result is expected since the concentrations of EDTA are large compared to the enzyme concentration. The apparent first-order rate constant in the presence of 10 μM EDTA is twice that in 5.0 μM EDTA (Fig. 3).

The binding site of EDTA

It can be seen in Figs 2 and 3 that formation of the enzyme–EDTA complex did not occur in the mutase reaction mixture. The EDTA inhibition was released under the inhibitory condition of Fig. 3 (10 μM EDTA in the preincubation, 20 μM EDTA in the mutase reaction) by addition of 2.0 mM glucose 1-phosphate and 12.5 μM glucose 1,6-diphosphate to the preincubation mixture. EDTA might bind to the enzyme competitively with the substrate or the coenzyme. The protective effect of various concentrations of the substrates and the coenzyme was examined to determine the dissociation constants of the binary complexes of the enzyme and the substrates or the coenzyme. Fig. 4 shows that the apparent first-order reaction constant was reduced on addition of the substrate or the coenzyme to the preincubation mixture.
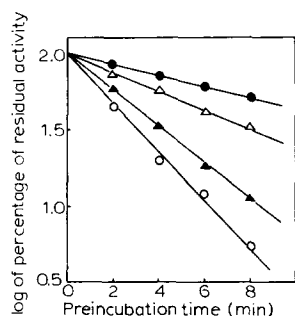

Fig. 4. Protective effects of the substrates and the coenzyme from the EDTA inhibition. The enzyme was preincubated with 10 μM EDTA in the presence or absence of the substrate and the coenzyme. The mutase reaction mixture contained 20 μM EDTA. Other conditions for the preincubation and the mutase reaction were same as in the legend to Fig. 3. Additions to the preincubation mixture: ○—○, none; △—△, 0.60 mM glucose 1-phosphate; ▲—▲, 0.20 mM glucose 6-phosphate; ●—●, 0.80 μM glucose 1,6-diphosphate.

In the absence of the ligand (the substrate or the coenzyme), the apparent first-order rate constant, k, is:

$$\frac{d[EI]}{dt} = k([E]_0 - [EI])$$

where E, I and EI represent the enzyme, EDTA and the enzyme–EDTA complex, respectively. In the presence of the ligand, the following equations are obtained on the assumption that the protective effect of the ligand is due to competition with EDTA:

$$\frac{d[EI]}{dt} = k([E]_0 - [EI] - [EL]),$$

$$\frac{([E]_0 - [EI]' - [EL])[L]}{[EL]} = K_s$$

where L and K_s represent the ligand and the dissociation constant of the enzyme–ligand complex, respectively. Thus the first-order reaction in the presence of the ligand is shown as follows:

$$\frac{d[EI]}{dt} = k \cdot \frac{K_s}{K_s + [L]} ([E]_0 - [EI])$$

The apparent first order rate constant in the presence of the ligand, k', is:

$$k' = k \cdot \frac{K_s}{K_s + [L]}$$

This indicates that the K_s value can be calculated from the values of k and k' obtained experimentally. Table I shows the K_s values for glucose 1-phosphate, glucose 6-phosphate and glucose 1,6-diphosphate obtained from this treatment. The important result in Table I is that the K_s value for glucose 1,6-diphosphate coincides well with the K_m^* value for the coenzyme obtained from kinetic experiments[9]. The K_s value for glucose 1-phosphate, however, does not agree with the K_m value for the substrate, but with the substrate inhibition constant, K_{si} (ref. 9). In the yeast phosphoglucomutase reaction, substrate inhibition occurred with the binding of substrate to the coenzyme binding site[9]. These observations suggest that the EDTA inhibition may be due to binding to the coenzyme binding site.

TABLE I

DISSOCIATION CONSTANTS OBTAINED FROM THE PROTECTIVE EFFECTS FROM EDTA INHIBITION

The enzyme activity was assayed after incubation at 25 °C in 10 mM Tris buffer (pH 7.5) with 10 μM EDTA in the presence or absence of ligands. The values of the kinetic experiments are from the data in the previous paper[9]. K_{si} represents the substrate inhibition constant.

Ligands	Protection experiments				Kinetics	
	Concn	k'/k	K_s		K_m (μM)	K_{si} (mM)
Glucose 1-phosphate	1.0 mM	0.23	0.3 mM	Average 0.5 mM	4.0	1.0
	0.6 mM	0.41	0.4 mM			
	0.1 mM	0.87	0.7 mM			
Glucose 6-phosphate	1.1 mM	0.29	0.5 mM	Average 0.5 mM	—	—
	0.2 mM	0.71	0.5 mM			
Glucose 1,6-diphosphate	10 μM	0.02	0.2 μM	Average 0.2 μM	0.14	—
	1.0 μM	0.16	0.2 μM			
	0.8 μM	0.23	0.2 μM			

Metal content of yeast phosphoglucomutase

The metal content of yeast phosphoglucomutase was assayed by atomic absorption spectrophotometry. The enzyme solution was dialyzed overnight against 10 mM citrate buffer (pH 5.3) containing 0.1 mM EDTA and was passed through a

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* As shown in two previous papers[9,10], the reaction of yeast phosphoglucomutase proceeds *via* a "rapid equilibrium random sequential" mechanism. K_m in the yeast phosphoglucomutase reaction represents the K_m value in the absence of a co-substrate. Thus, the K_m value is nearly equal to the dissociation constants between the enzyme and glucose 1-phosphate or glucose 1,6-diphosphate in the absence of a co-substrate.

Sephadex G-50 column equilibrated with the same buffer to remove extraneous metal ions. Table II shows that yeast phosphoglucomutase contains 0.87 mole of zinc, whereas the magnesium content, the essential metal for the muscle enzyme reaction, is negligibly low.

TABLE II

METAL CONTENT OF YEAST PHOSPHOGLUCOMUTASE

The enzyme solution was dialyzed overnight against 10 mM citrate buffer (pH 5.3) containing 0.1 mM EDTA, and was subjected to gel filtration on a Sephadex G-50 column (1.6 cm × 20 cm) which was equilibrated with the same buffer.

Metal	Observed metal (μM)	Concn of the enzyme (μM)	Mole of metal per mole of the enzyme
Zinc	0.93	1.10	0.84 ⎫
	1.15	1.30	0.88 ⎬ Average 0.87
	1.60	1.80	0.89 ⎭
Magnesium	<0.05	0.80	<0.06
		1.40	

Yeast phosphoglucomutase under neutral and acid conditions

The difference between the acid and the neutral enzyme shown in Figs 1 and 2 was studied with a hydrophobic probe, ANS. It was shown in the previous paper[10] that about eight ANS molecules were bound by the enzyme in neutral buffer. The number was identical in acid buffer, and did not change on addition of EDTA. However, the fluorescence intensity of ANS bound by the enzyme was quenched in a time-dependent manner by the addition of EDTA. Fig. 5 shows that the quenching proceeds as an apparent first-order reaction. In neutral solution, the half-life was 1.8 min. The half-life obtained from the inhibitory effect of the same concentration of EDTA was 1.9 min as shown in Fig. 3. This agreement suggests that the state of the hydrophobic region of the enzyme molecule is changed by the binding of EDTA. On
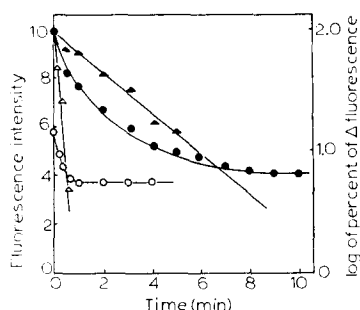

Fig. 5. The time-dependent quenching of the fluorescence of ANS induced by EDTA. To 2.0 ml of the enzyme solution containing 0.20 mM ANS and 20 mM ammonium sulfate in a cuvette, were added 10 μl of 2.0 mM EDTA. The decrease in fluorescence of ANS bound by the enzyme was measured in 10 mM Tris buffer (pH 7.5), (●—●), or in 10 mM acetate buffer (pH 5.3), (○—○). The values on the rigth ordinate were calculated from the ratio of each decrease relative to the maximum decrease of the fluorescence. ▲ and △ represent the value in neutral buffer and in acid buffer, respectively.

the other hand, the half-life of ANS quenching was about 10 s in acid solution. The EDTA–enzyme complex was formed in acid solution with a speed of one-order magnitude faster than in neutral solution.

DISCUSSION

It has been shown that intrinsic metal is essential for the yeast phosphoglucomutase reaction, and that EDTA inhibition was due to formation of the enzyme–metal–EDTA complex. About 1 mole of zinc, which was not removed by treatment with EDTA, was observed per mole of enzyme by atomic absorption measurements. This intrinsic zinc has not been conclusively shown to be essential for the catalytic activity of the yeast enzyme, since the criteria proposed by Vallee[18] for the identification of a metalloenzyme were not always fulfilled. However, it is unlikely that significant amounts of transition metals are contained by yeast phosphoglucomutase, since it did not show any absorption at visible wavelengths. Various enzymes[18] are known to be zinc-metalloenzymes. It has been reported that enzymes from yeast such as alcohol dehydrogenase[18], phosphomannose isomerase[19], fructose-1,6-diphosphate aldolase[20], and pyruvate carboxylase[21] are zinc-metalloenzymes. As pointed out by Scrutton *et al.*[21], metalloenzymes from yeast typically seem to contain zinc as the intrinsic metal ion. It was reported in previous papers[9,10] that the reaction of yeast phosphoglucomutase proceeded *via* a "rapid equilibrium random sequential" pathway. Thus the binding sites of the substrate and the coenzyme are considered to be different on the yeast enzyme surface. The protective effect of the coenzyme from the EDTA inhibition suggested that the intrinsic metal existed on the coenzyme binding site. In the previous paper[10], it was shown that 1 mole of coenzyme was bound by 1 mole of enzyme. This value agreed with the zinc content of the enzyme. These observations support the theory that the intrinsic zinc exists on the coenzyme binding site.

The difference between the yeast and the muscle enzyme in the stimulation by chelating agents can be explained by the fact that the yeast enzyme contained intrinsic metal whereas the muscle enzyme required extrinsic Mg^{2+} (see refs 11–16). Simultaneous addition of chelating agent and Mg^{2+} was required for the activation of the muscle enzyme[11–14]. It was indicated by Milstein[15,16] that the role of a chelating agent for the activation of the muscle enzyme is to bind inhibitory heavy-metal ions and hence to aid the addition of Mg^{2+} to the enzyme. Thus, cysteine was the best activator and EDTA the poorest, since the former has a high affinity for heavy metals and a low affinity for Mg^{2+}, while the latter has a high affinity for both metal ions[15,16]. However, the yeast enzyme activity could be stimulated by the addition of EDTA, regardless of the presence of a bivalent cation. This suggested that EDTA stimulation was due to the removal of inhibitory metal(s) attached to the different site(s) from that bound by the essential intrinsic metal.

The difference between Figs 1 and 2 may be explained as follows. The binding of EDTA to the yeast enzyme in neutral solution was relatively slow and was reduced by the presence of the coenzyme. Thus the enzyme activity is not inhibited by EDTA when the reaction is started by the addition of the enzyme solution dissolved in neutral buffer. On the other hand, in acid solution the enzyme was bound by EDTA at a relatively high speed. This difference between neutral and acid conditions is considered to be due to the difference in the state of the enzyme, since EDTA has a

higher affinity for free metal ions at the higher pH than at the lower pH. The EDTA inhibition shown in Fig. 1 may be due to the binding of EDTA to the acid-state enzyme during the conversion of the acid state to the neutral state of the enzyme.

It was reported by Ray[22] and Peck and Ray[23,24] that in the muscle enzyme reaction Ni^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+} were able to substitute for Mg^{2+} and that bivalent cations induced a conformational change in the enzyme molecule. They suggested from the values of the dissociation constants of the enzyme–metal complexes that muscle phosphoglucomutase might be classed as a "metalloenzyme" in the case of zinc and as a "metal-activated enzyme" with respect to magnesium[22]. It was also demonstrated that the zinc enzyme exerted only 0.3% activity of the magnesium enzyme, and Zn^{2+} acted as an inhibitor in the muscle enzyme reaction[22]. However, the specific activity of yeast phosphoglucomutase[9] did not show a great difference from that of the muscle enzyme saturated with Mg^{2+}. These differences in metal ion requirements between yeast and muscle phosphoglucomutase seem to be important for understanding the reaction sequences of mutases.

In the phosphoglucomutase reaction, a phosphate group of the coenzyme must be hydrolyzed. The hydrolyzed phosphate group must be directly transferred to the substrate in the "sequential" pathway of the yeast enzyme, whereas the phosphate group must be transferred to the enzyme to form the stable enzyme–phosphate complex in the "ping-pong" pathway of the muscle enzyme. It was shown in the muscle phosphoglucomutase reaction that the order of addition of Mg^{2+} and glucose 1-phosphate to the phospho-enzyme, and of Mg^{2+} and glucose 1,6-diphosphate to the dephospho-enzyme was random type[25]. According to the results of the report[25], the binding of Mg^{2+} to the muscle enzyme did not affect the binding of the substrate to the enzyme and slightly facilitated that of the coenzyme. These suggest that Mg^{2+} is not required for the binding of the substrate and the coenzyme but for the catalytic step of the muscle enzyme reaction. However, it is suggested in this report that the intrinsic zinc plays an important role in the binding of the coenzyme to the yeast phosphoglucomutase. It is considered that bivalent cations may have one of the important effects on the determination of the reaction mechanisms of phosphoglucomutases.

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