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# STUDIES ON A ROLE OF THE 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE ACTIVITY IN THE YEAST PHOSPHOGLYCERATE MUTASE REACTION

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SUMMARY

1. In order to clarify a role of the 2,3-diphosphoglycerate phosphatase activity catalyzed by yeast phosphoglycerate mutase (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3), chemical modification experiments and kinetic studies on the phosphatase activity have been performed. The results indicated that the substrate site of the mutase was also required for the phosphatase activity.

2. It was found that such compounds as phosphoglycolate, phosphohydroxypyruvate and phosphoenolpyruvate stimulated the phosphatase activity, although they inhibited the mutase activity. A kinetic pattern of their stimulatory effects was consistent with an equation derived from the ideas that a ternary complex (enzyme–2,3-diphosphoglycerate–2,3-diphosphoglycerate) was an active intermediate in the phosphatase reaction and that the activators interacted with the enzyme at the substrate site to stimulate the hydrolysis of a phosphoester bond in 2,3-diphosphoglycerate at the coenzyme site.

3. It was found that the phosphatase activity of component I (the native enzyme) was stimulated in higher extent than that of component V (the final product of the enzymic modification of component I). This observation offered a significant clue to explain a cause of the decrease in the mutase activity by the enzymic modification of component I to component V.

4. From these results, a hypothetical model for a role of the phosphatase activity on the mutase reaction mechanism is presented in this paper.

## INTRODUCTION

The phosphoglycerate mutase (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) preparations highly purified from yeast and

Abbreviations: 2PGA, 2-phospho-DL-glycerate; 3PGA, 3-phospho-D-glycerate; 2,3PGA, 2,3-diphospho-D-glycerate; PGA, phosphoglycerate.

rabbit muscle have been shown to exhibit the 2,3-diphosphoglycerate phosphatase activity[1,2], although the phosphatase activity was very low compared with the mutase activity. It was reported that $Hg^{2+}$ had a stimulatory effect on the phosphatase activity of the muscle enzyme and no effect on the yeast enzyme[3]. A previous report has suggested that the phosphatase activity in the yeast enzyme preparation is not due to a contaminated enzyme but to the phosphoglycerate mutase[2].

In order to obtain further evidence to support that both the mutase and the phosphatase activities in the yeast preparation are catalyzed by a single enzyme, chemical modification experiments by using trinitrobenzenesulfonate were performed. Furthermore, from the kinetic studies on the activation of the phosphatase activity by substrate analogues (phosphoglycolate, phosphohydroxypyruvate and phosphoenolpyruvate), a possible role of the phosphatase activity on the mutase reaction pathway is presented in this paper. It is also described that the decrease in the mutase activity by the enzymic modification[2] may be interpreted in terms of the decrease in the activation of the phosphatase activity.

## MATERIALS AND METHODS

### *Crystalline yeast phosphoglycerate mutase*

The methods for preparation of component I and V were the same as in the preceding paper[2,4].

### *Other materials*

3-Phospho-D-glycerate, 2-phospho-DL-glycerate, 2,3-diphospho-D-glycerate, phosphoenolpyruvate, phosphoglycolate, phosphohydroxypyruvate and trinitrobenzenesulfonate were as in the preceding paper[4]. The barium salts of phospho-DL-lactate were a kind gift from Dr. Pizer.

### *Enzyme activity*

The mutase activity was determined polarimetrically as described previously[5].

The reaction mixture for the phosphatase activity measurement contained components as described in legends of individual figures and tables in a total volume of 2 ml. After incubation at 25°, inorganic phosphate liberated from 2,3-diphosphoglycerate was determined. The reaction mixture was directly subjected to color development by the method of BARTLETT[6]. In Table I, the method of CHEN *et al.*[7] was used except that the method of MARSH[8] was used when ATP was added to the reaction mixture. $v$ in figures is expressed as $\mu$g of phosphorous liberated per min in the reaction mixture. The activity in all instances was measured under conditions such that initial reaction velocities were determined.

### *3- and 2-phosphoglycerate mixture*

The solution containing 3- and 2-phosphoglycerate at the concentrations in enzymic equilibrium was prepared as follows. The reaction mixture, in 15.5 ml, 20 mM 3-phosphoglycerate (pH 5.9), 10 $\mu$M 2,3-diphosphoglycerate (pH 5.9) and 876 $\mu$g of the mutase. After incubation for 2 h at 25°, the mixture was passed through Amberite IR-120 ion exchanger ($H^+$ form) to remove the enzyme protein. The eluted

TABLE I

EFFECTS OF VARIOUS COMPOUNDS ON PHOSPHOGLYCERATE MUTASE AND 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE ACTIVITIES

The reaction mixture for determination of the mutase activity contained, in 5 ml, 8 mM 2-phosphoglycerate, 0.1 mM 2,3-diphosphoglycerate, 0.1 M acetate buffer (pH 5.9) and 1.92 μg of the enzyme. After incubation at 25°, the activity was measured polarimetrically as described previously[5]. The reaction mixture for determination of the phosphatase activity contained, in 2 ml, 0.1 mM 2,3-diphosphoglycerate, 24 μg of the enzyme and 0.1 M acetate buffer (pH 5.9). The enzyme preparation had the activity of 60% of component I and contained all components (components I, II, III, IV, and V). Phosphate compounds in this table did not serve as a substrate of the phosphatase activity.

| *Compounds* | *Concn. (mM)* | *Phospho-glycerate mutase activity (%)* | *Concn. (mM)* | *2,3-Diphospho-glycerate phosphatase activity (%)* |
|---|---|---|---|---|
| No addition | — | 100 | — | 100 |
| 3-Phosphoglycerate | — | — | 0.20 | 62 |
| 2-Phosphoglycerate | — | — | 0.20 | 44 |
| Phosphoglycolate | 5.5 | 44 | 0.27 | 460 |
| Phosphohydroxypyruvate | 2.0 | 40 | 0.72 | 4260 |
| Phosphoenolpyruvate | 5.0 | 78 | 5.00 | 252 |
| Phospho-DL-lactate | 5.0 | 75 | 5.00 | 124 |
| Citrate | 5.0 | 70 | 5.00 | 316 |
| Isocitrate | 5.0 | 63 | 1.00 | 183 |
| ATP | 10.0 | 80 | 1.00 | 215 |

solution was adjusted to pH 5.9. The equilibrium constant at 25° was reported to be 5.2 ([3-phosphoglycerate]/[2-phosphoglycerate])[9].

*Other methods*

The procedures for trinitrophenylation of the enzyme and for determination of the enzyme concentration were as in the previous paper[4].

RESULTS

*Parallel loss of the phosphoglycerate mutase and the 2,3-diphosphoglycerate phosphatase activities by trinitrophenylation*

It has been reported in the preceding paper[4] that amino groups reactive to trinitrobenzenesulfonate are located at the substrate site of the mutase. As shown in Fig. 1, trinitrophenylation of the mutase inactivated both the mutase and phosphatase activities in almost the same manner, while 3-phosphoglycerate (the substrate of the mutase reaction) protected the phosphatase activity from the inactivation as well as the mutase activity. Phosphoglycolate, phosphohydroxypyruvate, phosphoenolpyruvate and citrate exhibited marked protective effects on the inactivation of the mutase activity[4]. The protection by these compounds against loss of the phosphatase activity was found to be the same as the protection against loss of the mutase activity. These observations offer further evidence for the idea that these

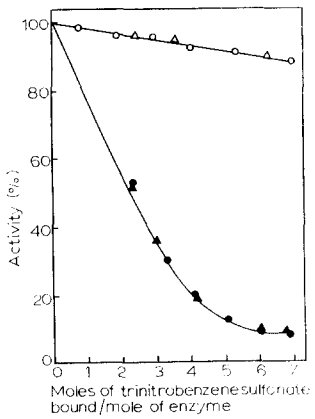



Fig. 1. The parallel loss of the phosphoglycerate mutase and the 2,3-diphosphoglycerate phosphatase activities by trinitrophenylation. ○, ●, the mutase activity; △, ▲, the phosphatase activity. ●, ▲, no 3-phosphoglycerate; ○, △, 2 mM 3-phosphoglycerate. The reaction mixture for trinitrophenylation contained 5 $\mu$M enzyme (component I) and 0.5 mM trinitrobenzenesulfonate in 25 mM borate buffer (pH 7.5). The temperature was 25°. At intervals samples were withdrawn to determine the activities and to determine the number of trinitrobenzenesulfonate bound per mole of the enzyme by measuring the absorbance at 348 nm as described in the preceding paper[4]. The reaction mixture for the determination of the phosphatase activity contained 0.5 mM 2,3-diphosphoglycerate and the trinitrophenylated enzyme (12 $\mu$g/ml) in 25 mM borate buffer (pH 7.5). The mutase activity was determined as described in Table I by using 2 $\mu$g of the enzyme.

two activities are due to a common enzyme. It is also concluded that the phosphatase activity may have an important role on the mutase reaction and that the substrate site of the mutase is essential for the mutase and the phosphatase activities.

*Effects of various compounds on phosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase activities*

Effects of various compounds on both the mutase and the phosphatase activities were summarized in Table I. Substrates (3- or 2-phosphoglycerate) of the mutase reaction gave a marked inhibitory effect on the phosphatase activity. It has been reported that the phosphate compounds (phosphoglycolate, phosphohydroxypyruvate and phosphoenolpyruvate) combine with the enzyme at the substrate site of the mutase to inhibit the mutase activity[4]. These compounds were found to exert a stimulatory effect on the phosphatase activity, although they all inhibited the mutase activity. These compounds did not serve as substrates for the phosphatase activity. It was found that citrate, isocitrate and ATP stimulated the phosphatase activity, while they inhibited the mutase activity. The compounds glycolate (5 mM), glycerate (0.5 mM), pyruvate (5 mM), hydroxypyruvate (5 mM), lactate (0.5 mM), DL-malate (5 mM), fumarate (5 mM), succinate (5 mM), itaconate (5 mM), glucose 6-phosphate (5 mM), fructose 1,6-diphosphate (0.5 mM), 6-phosphogluconate (5 mM) and EDTA (5 mM) did not affect both activities. However, oxalate (5 mM) inhibited 40% of the mutase activity, although the phosphatase activity was not affected.

PIZER AND BALLOU[10] indicated in the paper studied the substrate specificity of the muscle enzyme that the enol and hydrated forms of phosphohydroxypyruvate were acceptable of a phosphate group from 2,3-diphosphoglycerate. It seems to be unlikely that phosphoglycolate is an acceptor of a phosphate group. Comparative

studies on effects of phosphoglycolate and phosphohydroxypyruvate may offer an important information on a role of the phosphatase activity.

Recently, ROSE AND LIEBOWITZ[11,12] reported that phosphoglycolate stimulated the 2,3-diphosphoglycerate phosphatase activity involved in the muscle phosphoglycerate mutase and that this stimulatory effect could be utilized for the determination of amounts of 2,3-diphosphoglycerate in tissues.

*Effect of 3-phosphoglycerate(2-phosphoglycerate) on the phosphatase activity*

The mode of an inhibitory effect of 3- and 2-phosphoglycerate on the phosphatase activity was investigated kinetically. The solution containing 3- and 2-phosphoglycerate in equilibrium was used to keep a constant concentration of the inhibitor. Inhibition was of the competitive type with 2,3-diphosphoglycerate as indicated by the common point of intersection of all plots on the $1/v$ axis (Fig. 2). An inhibition constant was found to be 0.24 mM. This value coincided approximately with the

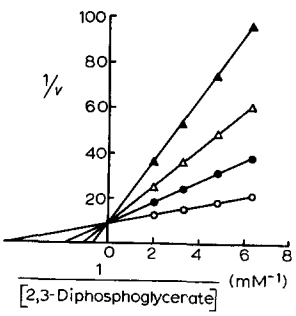



Fig. 2. Inhibition of the 2,3-diphosphoglycerate phosphatase activity by equilibrated 3- and 2-phosphoglycerate. The reaction mixture contained, in 2 ml, 74 $\mu$g of the enzyme (component I), 0.1 M acetate buffer (pH 5.9), the indicated concentrations of 2,3-diphosphoglycerate and either no (○), 0.4 mM (●), 0.8 mM (△), or 1.6 mM equilibrated 3- and 2-phosphoglycerate (▲).

value of $K_m^{3PGA}$ in the mutase reaction[13]. The $K_m^{2,3PGA}$ value of 0.22 mM in the absence of the inhibitor seems to indicate a Michaelis constant at a different position from the coenzyme site of the mutase, since the value of $K_m^{2,3PGA}$ in the mutase reaction has been reported to be 0.8 $\mu$M[13]. It was found that 2,3-diphosphoglycerate at a high concentration in the mutase reaction was a competitive inhibitor of the substrate and that the value of $K_i^{2,3PGA}$ was 0.2–0.5 mM[4]. From these informations, it is assumed that the $K_m^{2,3PGA}$ value in the phosphatase reaction is a Michaelis constant of 2,3-diphosphoglycerate at the substrate site of the mutase. This is supported by the facts that amino groups essential for binding of the substrate were also required for the phosphatase activity (Fig. 1) and that a higher concentration of 2,3-diphosphoglycerate than 1 mM was required for protection of the enzyme from inactivation by trinitrophenylation[4]. A competitive type inhibition by the mixture of 3- and 2-phosphoglycerate means that a competitive binding of these compounds with 2,3-diphosphoglycerate occurs at the substrate site of the mutase (Fig. 2).

TABLE II

ACTIVATION OF THE 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE ACTIVITIES OF COMPONENT I AND V

The reaction mixture contained, in 2 ml, 0.2 mM 2,3-diphosphoglycerate, 37 $\mu$g of the enzyme (component I or V), 0.1 M acetate buffer (pH 5.9) or 25 mM borate buffer (pH 7.5) and the indicated concentrations of phosphoglycolate or phosphohydroxypyruvate. Activity is expressed as $\mu$g of phosphorous liberated per min under the above conditions.

| *Compound added* | *Concn. (mM)* | *Activity* | | | |
|---|---|---|---|---|---|
| | | *Component I* | | *Component V* | |
| | | *pH 5.9* | *pH 7.5* | *pH 5.9* | *pH 7.5* |
| No addition | — | 0.026 | 0.024 | 0.025 | 0.024 |
| Phosphoglycolate | 1.0 | 0.270 | 0.480 | 0.050 | 0.084 |
| Phosphohydroxypyruvate | 0.1 | 0.490 | 0.740 | 0.097 | 0.112 |

*Effects of phosphoglycolate and phosphohydroxypyruvate on the phosphatase activity*

As shown in Table II, component V had almost the same phosphatase activity as that of component I, although the mutase activity of component V was about 5% of component I[2]. However, it was found that the phosphatase activity of component I was stimulated by the phosphate compounds in higher extent than that of component V. A detailed kinetic investigation of the stimulatory effect on the phosphatase activity was also performed on both components.

Activation of the phosphatase activities of component I and V by phosphoglycolate and phosphohydroxypyruvate was found to be dependent on pH. The

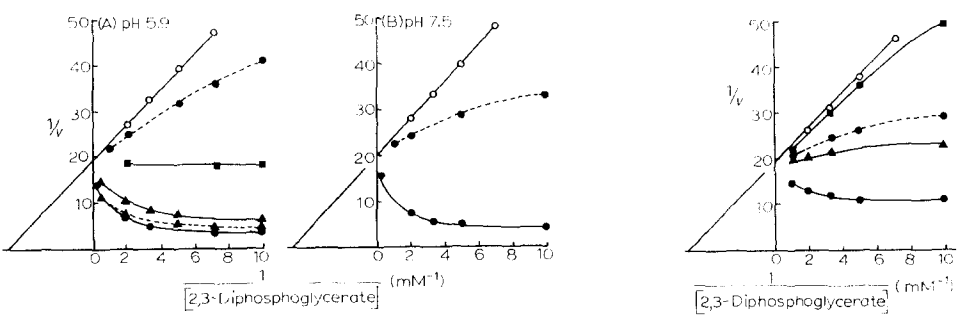



Fig. 3. Effects of phosphoglycolate and phosphohydroxypyruvate on the 2,3-diphosphoglycerate phosphatase activity of component I. The reaction mixture of (A) contained, in 2 ml, 37 $\mu$g of the enzyme (component I), 0.1 M acetate buffer (pH 5.9), the indicated concentrations of 2,3-diphosphoglycerate and either no (—○—), 11.2 $\mu$M phosphoglycolate (– – –●– – –), 0.54 mM phosphoglycolate (– – –▲– – –), 4.5 $\mu$M phosphohydroxypyruvate (—■—), 18 $\mu$M phosphohydroxypyruvate (—▲—), or 36 $\mu$M phosphohydroxypyruvate (—●—). The reaction mixture of (B) contained, in 2 ml, 37 $\mu$g of the enzyme (component I), 25 mM borate buffer (pH 7.5), the indicated concentrations of 2,3-diphosphoglycerate and either no (—○—), 10 $\mu$M phosphoglycolate (– – –●– – –), or 20 $\mu$M phosphohydroxypyruvate (—●—).

Fig. 4. Effects of phosphoglycolate and phosphohydroxypyruvate on the 2,3-diphosphoglycerate phosphatase activity of component V. The reaction mixture contained, in 2 ml, 37 $\mu$g of the enzyme (component V), 0.1 M acetate buffer (pH 5.9), the indicated concentrations of 2,3-diphosphoglycerate, and either no (—○—), 0.27 mM phosphoglycolate (– – –●– – –), 2 $\mu$M phosphohydroxypyruvate (—■—), 20 $\mu$M phosphohydroxypyruvate (—▲—), or 80 $\mu$M phosphohydroxypyruvate (—●—).

values of pH in Table II were selected from the facts that phosphoglycerate mutase showed the maximal activity at pH 5.9[9] and that the chemical modification experiments with trinitrobenzenesulfonate were performed at pH 7.5[4].

As shown in Figs. 3 and 4, Lineweaver–Burk plots of the phosphatase activities in the presence of the activator were nonlinear. A similar pattern was observed in both components. The phosphatase activity was decreased progressibely as the concentration of 2,3-diphosphoglycerate was increased, when an activator was given at a high concentration. On the other hand, at a dilute concentration of an activator, the activity was increased. In the absence of an activator, the values of $K_m^{2,3PGA}$ were almost the same in both components and no alteration was observed at pH tested.

Phosphoenolpyruvate exhibited its stimulatory effect in a similar manner to that of phosphoglycolate or phosphohydroxypyruvate.

Based on the results indicating that the binding of 2,3-diphosphoglycerate at the substrate site of the mutase was required for exhibition of the phosphatase activity and that activators combined with the enzyme at the substrate site of the mutase, the most plausible mechanism that may explain the data of Figs. 3 and 4 was described in the following.

$$E + 2,3PGA \rightleftharpoons E \cdot 2,3PGA_1 \qquad K_1^{2,3PGA} \qquad (1)$$

$$E + 2,3PGA \rightleftharpoons E \cdot 2,3PGA_2 \qquad K_2^{2,3PGA} \qquad (2)$$

$$E \cdot 2,3PGA_1 + 2,3PGA \rightleftharpoons E \cdot 2,3PGA_1 \cdot 2,3PGA_2 \qquad K_2^{2,3PGA} \qquad (3)$$

$$E \cdot 2,3PGA_2 + 2,3PGA \rightleftharpoons E \cdot 2,3PGA_2 \cdot 2,3PGA_1 \qquad K_1^{2,3PGA} \qquad (4)$$

$$E \cdot 2,3PGA_1 \cdot 2,3PGA_2 \xrightarrow{k} E \cdot 2,3PGA_2 + Pi + 3 \text{ or } 2PGA$$
$$(E \cdot 2,3PGA_2 \cdot 2,3PGA_1) \qquad (5)$$

$$E + A \rightleftharpoons E \cdot A \qquad K^A \qquad (6)$$

$$E \cdot 2,3PGA_1 + A \rightleftharpoons E \cdot 2,3PGA_1 \cdot A \qquad K^A \qquad (7)$$

$$E \cdot A + 2,3PGA \rightleftharpoons E \cdot A \cdot 2,3PGA_1 \qquad K_1^{2,3PGA} \qquad (8)$$

$$E \cdot 2,3PGA_1 \cdot A \xrightarrow{ak} E \cdot A + P_i + 3 \text{ or } 2PGA$$
$$(E \cdot A \cdot 2,3PGA_1) \qquad (9)$$

$$v_0 = k[E \cdot 2,3PGA_1 \cdot 2,3PGA_2] = k[E \cdot 2,3PGA_2 \cdot 2,3PGA_1]$$
$$V = k[E]_t$$
$$v_a = v_0 + ak[E \cdot 2,3PGA_1 \cdot A] = v_0 + ak[E \cdot A \cdot 2,3PGA_1]$$

Here, $E \cdot 2,3PGA_1$ represents an enzyme species which binds 2,3-diphosphoglycerate at the coenzyme site of the mutase and $E \cdot 2,3PGA_2$ represents an enzyme species which binds 2,3-diphosphoglycerate at the substrate site of the mutase. Dissociation constants of 2,3-diphosphoglycerate at the coenzyme site and the substrate site are expressed with $K_1^{2,3PGA}$ and $K_2^{2,3PGA}$, respectively. A symbol of $K^A$ represents a dissociation constant of an activator, A, at the substrate site. A rate constant is represented by $k$ and an activation effect, by $a(a > 1)$.

In this mechanism, the following assumption is made. The enzyme species of $E \cdot 2,3PGA_1$ hydrolyses a phosphoester bond in 2,3-diphosphoglycerate with a very

low rate in comparison with the enzyme species of $E \cdot 2,3PGA_1 \cdot 2,3PGA_2$ or does not hydrolyse the bond. The cleavage of a phosphoester bond proceeds at the coenzyme site to produce 1 mole of inorganic phosphate per mole of 2,3-diphosphoglycerate when the complex of $E \cdot 2,3PGA_1 \cdot 2,3PGA_2$ is formed.

A value of $K_m^{2,3PGA}$ in the mutase reaction corresponds to that of $K_1^{2,3PGA}$. The value of $K_m^{2,3PGA}$ was found to be 0.8 $\mu$M[13]. Therefore, the coenzyme site is completely saturated with 2,3-diphosphoglycerate under the conditions ([2,3-diphosphoglycerate] $\geqq$ 0.1 mM) employed in this paper. Under this condition, the steps, 1, 2, 4, 6 and 8 in the mechanism can be excluded. All equations were derived by the rapid equilibrium treatment. The equation representing the initial velocity ($v_0$) in the absence of an activator is,

$$v_0 = \frac{V[2,3PGA]}{K_2^{2,3PGA} + [2,3PGA]} \tag{1}$$

where $V$ is the maximal velocity in the absence of an activator. The equation in the presence of an activator is

$$v_a = \frac{V(K^A[2,3PGA] + \alpha K_2^{2,3PGA}[A])}{K_2^{2,3PGA}K^A + K^A[2,3PGA] + K_2^{2,3PGA}[A]} \tag{2}$$

Theoretical Lineweaver–Burk plots of Eqn. 2 are shown in Fig. 5. Patterns at different concentrations of an activator are consistent with those of Figs. 3 and 4. The theoretical lines drawn using the constants (Table III) which were determined from Figs. 6 and 7 coincided with the lines in Figs. 3 and 4.

In order to estimate quantitatively kinetic parameters of $K^A$ and $\alpha$, experi-

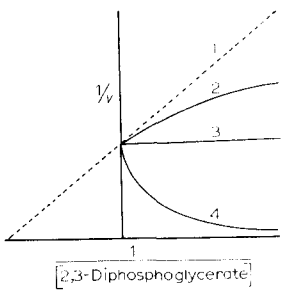



Fig. 5. Theoretical Lineweaver–Burk plots of Eqn. 2. —, in the presence of an activator; – – – –, in the absence of an activator. 1, [A] = 0; 2, [A] $< K^A/(\alpha - 1)$; 3, [A] = $K^A/(\alpha - 1)$; 4, [A] > $K^A/(\alpha - 1)$.

ments varying the concentration of an activator at a fixed concentration of 2,3-diphosphoglycerate were performed. From Eqns. 1 and 2, plotting $1/(v_a - v_0)$ to 1/[A] at a fixed concentration of 2,3-diphosphoglycerate should give linear curves. The values of $K^A$ and $\alpha$ can be calculated from the intersecting point on the 1/[A] axis and that on the $1/(v_a - v_0)$ axis, respectively. The results obtained at the constant 2,3-diphosphoglycerate concentration of 0.2 mM and different concentrations of an activator are shown in Figs. 6 and 7. Linear curves in both components were obtained independently on a kind of activators and pH incubated.

TABLE III

KINETIC PARAMETERS OF PHOSPHOGLYCOLATE AND PHOSPHOHYDROXYPYRUVATE

Kinetic parameters were calculated from Figs. 6 and 7.

| *Component* | *Phosphoglycolate* | | | | *Phosphohydroxypyruvate* | | | |
|---|---|---|---|---|---|---|---|---|
| | *pH 5.9* | | *pH 7.5* | | *pH 5.9* | | *pH 7.5* | |
| | $K^A$ *(mM)* | $\alpha$ | $K^A$ *(mM)* | $\alpha$ | $K^A$ *(mM)* | $\alpha$ | $K^A$ *(mM)* | $\alpha$ |
| I | 0.5 | 9.8 | 0.42 | 18 | 0.13 | 32 | 0.13 | 54 |
| V | 0.5 | 1.5 | 0.5 | 3 | 0.13 | 5.3 | 0.19 | 8.2 |

As summarized in Table III, phosphohydroxypyruvate was found to be a more effective activator with about 3-fold activating effect as compared with phosphoglycolate. Activation depended on the pH incubated, although the values of $K^A$ were not influenced. The most important result is that the extent of activation ($\alpha$) was different in components I and V.

*Evidence for competitive binding of 3-phosphoglycerate (2-phosphoglycerate),2,3-diphosphoglycerate and an activator at the substrate site of the mutase*

The competitive binding of 3-phosphoglycerate (2-phosphoglycerate), 2,3-di-

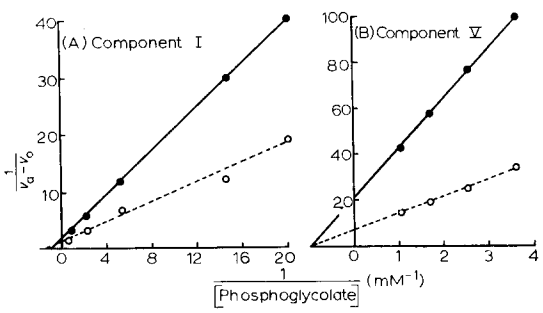



Fig. 6. Effects of phosphoglycolate on 2,3-diphosphoglycerate phosphatase activities of component I and V. The reaction mixture of (A) contained, in 2 ml, 0.2 mM 2,3-diphosphoglycerate, 37 $\mu$g of the enzyme (component I), the indicated concentrations of phosphoglycolate and either 0.1 M acetate buffer (pH 5.9) (●) or 25 mM borate buffer (pH 7.5) (○). The reaction mixture of (B) was the same as (A) except that component V was used as the enzyme. $v_0$, the initial velocity in the absence of phosphoglycolate; and $v_a$, the initial velocity in the presence of phosphoglycolate were expressed as $\mu$g of phosphorous liberated per min in the reaction mixture.

phosphoglycerate and an activator at the substrate site of the mutase was also verified kinetically by the following studies on the phosphatase reaction. The phosphatase activity stimulated by phosphoglycolate or phosphohydroxypyruvate should be reduced by the substrate (3- and 2-phosphoglycerate). The equation represents the initial velocity ($v$) of the phosphatase activity in the presence of 3-phosphoglycerate (2-phosphoglycerate), 2,3-diphosphoglycerate and an activator can be derived with the additional incorporation of an enzyme species, $E \cdot 2,3PGA_1 \cdot PGA$, to the mecha-

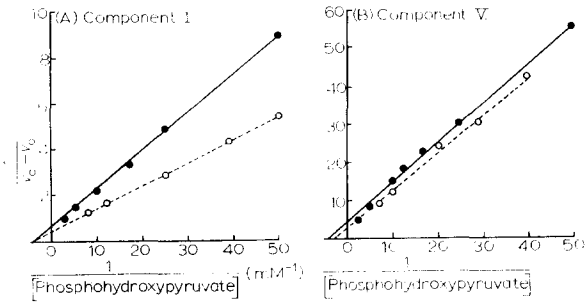



Fig. 7. Effects of phosphohydroxypyruvate on 2,3-diphosphoglycerate phosphatase activities of component I and V. The conditions for determination of the activity and symbols in figures were the same as in Fig. 6 except that phosphohydroxypyruvate instead of phosphoglycolate was used as an activator.

nism described above. In this case the mixture of 3- and 2-phosphoglycerate was expressed by PGA. The equation is

$$v = \frac{VK_i^{PGA}(K^A[2,3PGA] + \alpha K_2^{2,3PGA}[A])}{K_2^{2,3PGA}K^AK_i^{PGA} + K^AK_i^{PGA}[2,3PGA] + K_2^{2,3PGA}K_i^{PGA}[A] + K_2^{2,3PGA}K^A[PGA]} \quad (3)$$

where all symbols are identical to the mechanism above described except that $K_i^{PGA}$ representing a dissociation constant of phosphoglycerate to the complex of $E \cdot 2,3PGA_1$· PGA is introduced. Plotting $1/v$ *versus* [phosphoglycerate] at different concentrations of an activator and a fixed concentration of 2,3-diphosphoglycerate gives linear lines

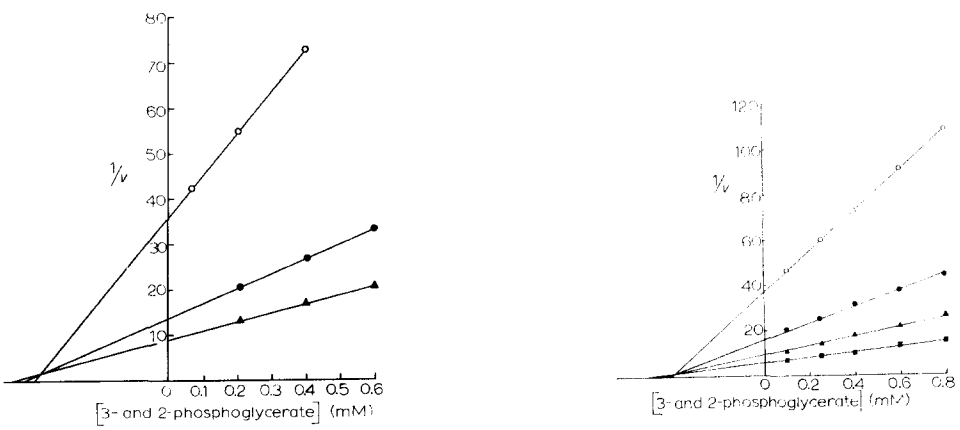



Fig. 8. Effects of equilibrated 3- and 2-phosphoglycerate on the activation of the 2,3-diphosphoglycerate phosphatase activity by phosphoglycolate. The reaction mixture contained, in 2 ml, 0.2 mM 2,3-diphosphoglycerate, 37 $\mu$g of the enzyme (component I), 0.1 M acetate buffer (pH 5.9), the indicated concentrations of equilibrated 3- and 2-phosphoglycerate and either no (○), 0.1 mM phosphoglycolate (●) or 0.2 mM phosphoglycolate (▲).

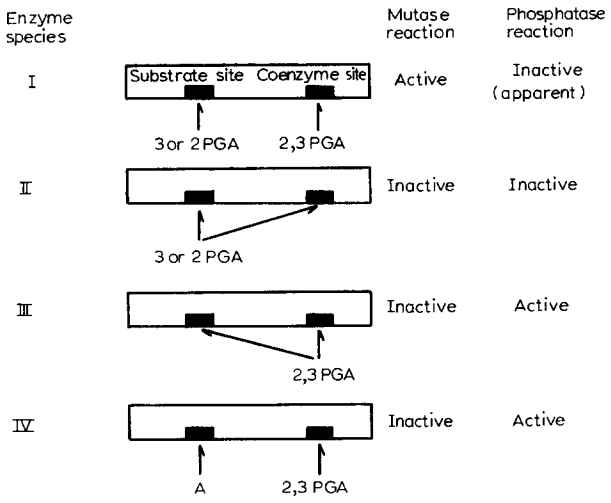
Fig. 9. Effects of equilibrated 3- and 2-phosphoglycerate on the activation of the 2,3-diphosphoglycerate phosphatase activity by phosphohydroxypyruvate. The conditions were the same as in Fig. 8 except that phosphohydroxypyruvate instead of phosphoglycolate was used. No phosphohydroxypyruvate (○), 10 $\mu$M phosphohydroxypyruvate (●), 20 $\mu$M phosphohydroxypyruvate (▲), 40 $\mu$M phosphohydroxypyruvate (■).

having a meet point in second quadrant. The concentration of 2,3-diphosphoglycerate was fixed to be 0.2 mM. The results at different concentrations of phosphoglycolate and phosphohydroxypyruvate are shown in Figs. 8 and 9 indicating that the velocity of the phosphatase activity is changed in the manner being fit for Eqn. 3. This observation is consistent with the idea that 3- and 2-phosphoglycerate repress the activation of the phosphatase activity by phosphoglycolate or phosphohydroxypyruvate through interactions with a common site on the enzyme.

The values of $K_i^{PGA}$ and $\alpha$ can be also calculated from Figs. 8 and 9. The $K_i^{PGA}$ value (0.2 mM) was well consistent with the value obtained from Fig. 2. The values of $\alpha$ ($\alpha = 12$ for phosphoglycolate and $\alpha = 35$ for phosphohydroxypyruvate) agreed with those of Table III ($\alpha = 9.8$ for phosphoglycolate and $\alpha = 32$ for phosphohydroxypyruvate).

## DISCUSSION

Kinetic analyses of the 2,3-diphosphoglycerate phosphatase activity which is exhibited by yeast phosphoglycerate mutase have been investigated. In order to explain a role of the phosphatase activity in the mutase reaction pathway, a hypothetical mechanism incorporating the results in this paper and in the previous papers[4,13] is presented in highly schematic form in Scheme 1.




Scheme 1. A hypothetical schematic model on a role of 2,3-diphosphoglycerate phosphatase activity on the phosphoglycerate mutase reaction pathway.

It seems possible to explain observations in this paper by a ping pong mechanism[14]. It should be noted, however, that this mechanism seems to be unlikely in the yeast mutase reaction pathway as described in the previous paper[4,13].

Scheme 1 is proposed on the basis of the following observations.

(1) Kinetic studies of the mutase activity[13] and chemical modification experiments[4] indicated that an enzyme species I in Scheme 1 is an active intermediate in the yeast mutase reaction pathway. As shown by an enzyme species II, the substrate

at a high concentration combines with the coenzyme site to inhibit the mutase activity[13].

(2) The type of inhibition observed at a high concentration of the coenzyme in the mutase reaction[4] indicates that the coenzyme can combine with the enzyme at the substrate site as shown by an enzyme species III. This was supported by the chemical modification experiments[4]. Amino groups essential for the substrate binding in the mutase reaction were also required for the phosphatase activity (Fig. 1). A value of $K_m^{2,3PGA}$ (0.22 mM) in the phosphatase reaction (Fig. 2) was very much larger than that of $K_m^{2,3PGA}$ (0.8 $\mu$M) in the mutase reaction and agreed with that of $K_i^{2,3PGA}$ (0.2–0.5 mM) in the mutase reaction. The mixture of 3- and 2-phosphoglycerate functioned as competitive inhibitor of 2,3-diphosphoglycerate in the phosphatase reaction (Fig. 2). From these observations, it may be concluded that an enzyme species III is an active intermediate in the phosphatase reaction. The coenzyme site was completely saturated with 2,3-diphosphoglycerate under the experimental conditions ($[2,3\text{-diphosphoglycerate}] \geqq 0.1$ mM) employed for the determination of the phosphatase activity, since the value of $K_m^{2,3PGA}$ in the mutase reaction was very small.

(3) Such phosphate compounds as phosphoglycolate, phosphohydroxypyruvate and phosphoenolpyruvate gave a stimulatory effect on the phosphatase activity. In the mutase reaction, these compounds were competitive inhibitors of the substrate[4]. And these compounds inhibited trinitrophenylation of amino groups essential for the substrate binding. From these results, it might be concluded that activators combined with the enzyme at the substrate site. Furthermore, the mode of a stimulatory effect on the phosphatase activity was consistent with such idea that an enzyme species III was an active intermediate in the phosphatase reaction and activators exhibited their effects through interactions with the enzyme at the substrate site as shown by an enzyme species IV. These results indicate that the cleavage of a phosphoester bond in 2,3-diphosphoglycerate occurs at the coenzyme site.

From this model, it may be mentioned that the first step in the mutase reaction is the hydrolysis of a phosphoester bond in 2,3-diphosphoglycerate at the coenzyme site. However, this hydrolysis proceeds at first by the formation of an enzyme–substrate–coenzyme complex. A spatial conformation of the enzyme required for hydrolysis of 2,3-diphosphoglycerate may be induced by binding of the substrate. However, acceptance of the phosphate group by the substrate results in proceeding of the mutase reaction and causes apparent inhibition of the phosphatase activity. Such compounds as 2,3-diphosphoglycerate (bound at the substrate site), phosphoglycolate, phosphohydroxypyruvate, and phosphoenolpyruvate contribute to induction of this conformational change through interactions with the substrate site. It was found that phosphohydroxypyruvate was more potential activator than phosphoglycolate. It is likely that an activator having high similarity to the substrate may induce a more favourable conformation for the phosphatase activity. And the most efficient activator may be 3- or 2-phosphoglycerate.

It was found that different activation ($\alpha$) was exerted on the phosphatase activities of component I and V, although $K^A$ values of activators were almost the same in both components (Table III). It was reported that difference in the specific activity of each component was not due to alteration in $K_m$ values for the substrate and the coenzyme but to alteration in the maximal activity[15]. The difference between

component I and V in the mutase activity may be ascribed to the difference in stimulatory effects (the values of $\alpha$ in Table III) on the phosphatase activities. The less effective induction of a spatial conformation in component V may decrease the efficient hydrolysis of 2,3-diphosphoglycerate and consequently the mutase activity. However, the difference between component I and V in the value of $\alpha$ did not correspond to that in the mutase activity, since the mutase activity of component V was about 5% of component I[2]. When the substrate combines at the substrate site, the difference between component I and V in the rate of hydrolysis of 2,3-diphosphoglycerate may correspond to that in the mutase activity.

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