Phosphoglycerate Mutase

KINETICS AND EFFECTS OF SALTS ON THE MUTASE AND BISPHOSPHOGLYCERATE PHOSPHATASE ACTIVITIES OF THE ENZYME FROM CHICKEN BREAST MUSCLE

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The steady state kinetics and effects of salts on chicken breast muscle phosphoglycerate mutase have been examined. The enzyme can catalyze three phosphoryl transfer reactions: mutase, bisphosphoglycerate phosphatase, and bisphosphoglycerate synthase. The mutase reaction was studied in the presence of the substrates, glycerate-2-P, glycolate-2-P, and bisphosphoglycerate-2-P. The latter is an analog of glycerate-3-P. The equilibrium between enzyme and glycerate-1,3-P$_2$ is favorable ($K_{eq}$ $\leq 7 \times 10^{-4}$ M) and suggests that in the absence of a separate synthase this reaction may have functional significance.

An enzyme that interconverts glycerate-3-P and glycerate-2-P, now designated phosphoglycerate mutase, was described by Meyerhof and Kiesling in 1935 (1). Sutherland et al. (2) observed that the rate of the reaction was enhanced by glycerate-2,3-P$_2$ and that there was isotopic mixing of phosphoryl groups of glycerate-3-P and those of the bisphosphate. More recently, we showed that glycerate-2,3-P$_2$ can donate a phosphoryl group to muscle or yeast phosphoglycerate mutase to form a stable covalent bond to histidine (3-5). There is kinetic evidence that the phosphoenzyme participates in the catalytic reaction (6). The structure of yeast phosphoglycerate mutase is being studied by x-ray crystallography (7, 8), and the amino acid sequence is being determined (8, 9).

In spite of the impressive increase in knowledge about the enzyme, there is no agreement as yet about the kinetic mechanism. Chiba et al. (10) studied the yeast enzyme and proposed a sequential pathway for the reaction. Grisolia and Cleland (11) concluded that the muscle enzyme has a ping-pong mechanism. The assay used in both studies was unsatisfactory because of the unfavorable equilibrium of the reaction in the direction of the assay (glycerate-3-P $\rightarrow$ glycerate-2-P) and the lack of sufficient sensitivity. Although Grisolia and Cleland (11) recognized that salts affect the rate of the enzymatic reaction, their experiments were poorly controlled with respect to salt concentrations. From a computer analysis, Mantle and Garfinkel (12) concluded that the data of Grisolia and Cleland did not allow an unambiguous choice of mechanism. We have now examined the steady state kinetics of phosphoglycerate mutase under carefully controlled conditions. To study the mutase reaction we have developed a sensitive new assay system that uses tritium-labeled glycerate-2-P as substrate in mixtures containing only the desired components, i.e. employing no linked assay system.

The glycerate-2,3-P$_2$-dependent phosphoglycerate mutase can catalyze three reactions (6, 13) which, will be designated as phosphoglycerate mutase (Equation 1), bisphosphoglycerate phosphatase (Equation 14-16), and bisphosphoglycerate synthase (Equation 17), and suggested in the absence of a separate synthase: this reaction may have functional significance.
Kinetic Properties of Phosphoglycerate Mutase

### MATERIALS AND METHODS

For the preparation of $[^2T]$glycerate-2-P, the following were lyophilized: imidazole-$\cdot$Cl, pH 6.0 (5 $\mu$mol); MgCl$_2$ (0.2 $\mu$mol); enolpyruvate-$P$ (10 $\mu$mol). Enolase (Boehringer 4 $\mu$l, 0.32 unit) and 0.1 ml of TOH (100 mCi) were added and the container was covered tightly for 14 h at room temperature. The reaction was stopped with 10 mM HCl. The glycerate-2-P was purified on a Dowex 1-Cl$^-$ column from which it eluted with 0.02 N HCl. Tritiated compounds were counted as gels under the assay conditions.

### Kinetic Studies

Phosphoglycerate Mutase Activity: $[^2T]$glycerate-2-P as Substrate—$[^2T]$glycerate-2-P that has been specifically labeled with tritium in the enolase reaction is used as the substrate. It is incubated with phosphoglycerate mutase under carefully controlled conditions (Equation 4). The reaction is stopped and the mutase inactivated, after which enolase and Mg$^{2+}$ are added to remove the tritium from the unreacted glycerate-2-P as TOH (Equation 5). The radioactivity in the $[^2T]$glycerate-3-P is then determined.

### RESULTS

Bisphosphoglycerate Phosphatase Activity

Lack of Bisphosphoglycerate Phosphatase Activity in the Absence of Effectors—Preparations of phosphoglycerate mutase have been reported to have phosphatase activity specific for glycerate-2,3-P$_2$ that is $10^{-4}$ to $10^{-5}$ times that of the mutase activity (20). For both the yeast and muscle enzymes, considerable enhancement of the phosphatase activity has been brought about by substances such as pyrophosphate (27, 28), glycolate-2-P (16, 22, 29), and phosphohydroxypyruvate (16, 30), all of which may be considered structural analogues of glycerate-2-P and glyceraldehyde-3-P. In order to evaluate the various potentiators of the phosphatase activity, it was necessary to determine accurately the nonactivated rate. However,
ever, when glycolate-2-P was omitted from the phosphatase assay system, there was no detectable rate of hydrolysis. An initial burst of $P_i$ was observed within the first 10 s and no additional $P_i$ release occurred during the following 10 min (Table IA). We showed previously (6) that under these conditions the enzyme is phosphorylated by glycerate-2,3-P$_2$ at $>100$ s$^{-1}$ forming an acid-labile phosphohistidine that functions catalytically in all reactions carried out by the enzyme. The $P_i$ released in the burst is equal to the amount of enzyme present (Table IB). These studies confirm the intimate relationship between the phosphatase and mutase activities and implicate the same active site (see also Sasaki et al. (16)).

**Effectors of the Phosphatase Activity**—A number of salts increase the phosphatase activity of phosphoglycerate mutase. Although 50 mM KCl alone has no detectable effect, it approximately doubles the stimulation of phosphatase activity that is induced by 5 mM potassium phosphate (Table II).

### Table I

**Absence of phosphatase activity in the nonactivated system**

<table>
<thead>
<tr>
<th>Time</th>
<th>cpm</th>
<th>P$_i$ released</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 s</td>
<td>2416</td>
<td>$1.55 \times 10^{-5}$</td>
</tr>
<tr>
<td>1 min</td>
<td>2588</td>
<td>$1.66 \times 10^{-5}$</td>
</tr>
<tr>
<td>5 min</td>
<td>2628</td>
<td>$1.68 \times 10^{-5}$</td>
</tr>
<tr>
<td>10 min</td>
<td>2490</td>
<td>$1.60 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

**Effectors of the phosphatase activity of phosphoglycerate mutase**

### Table II

<table>
<thead>
<tr>
<th>Effectors</th>
<th>P$_i$ released</th>
<th>µmol/min/ml of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KCl (50 mM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Potassium phosphate (5 mM)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>KCl (50 mM) + potassium phosphate (5 mM)</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>B. Glycolate-2-P (1 mM)</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td>Glycolate-2-P (1 mM) + KCl (50 mM)</td>
<td>49.3</td>
<td></td>
</tr>
<tr>
<td>Glycolate-2-P (1 mM) + potassium phosphate (5 mM)</td>
<td>40.4</td>
<td></td>
</tr>
<tr>
<td>Glycolate-2-P (1 mM) + potassium phosphate (5 mM) + KCl (50 mM)</td>
<td>43.5</td>
<td></td>
</tr>
</tbody>
</table>

Glycolate-2-P is a far more potent activator of the phosphatase activity. The phosphatase rate with suboptimal levels of substrate and glycolate-2-P is more than doubled by addition of 0.05 M KCl or 5 mM potassium-phosphate. The two salts together give no additional effect although the maximal velocity for the glycolate-2-P-activated system is more than twice the rates being observed. Subsequent studies will serve to clarify these observations.

**Kinetics of the Phosphatase Reaction in the Presence of Glycolate-2-P**—When glycerate-2,3-P$_2$ was varied at several levels of glycolate-2-P, the double reciprocal plots shown in Fig. 1 were obtained. In this analysis, glycolate-2-P is treated as a substrate although it does not undergo chemical change. At low concentrations of glycerate-2,3-P$_2$, glycolate-2-P is an inhibitor. The data in Fig. 1 were analyzed with those from a total of five similar experiments by computer using nonlinear regression analysis for mechanisms designated in the terminology of Cleland (31): 1) Ping-Pong Bi-Bi, where Bi-Bi refers to the participation of two substrates and Ping-Pong to the occurrence of an irreversible step (i.e. phosphorylation of the enzyme) before the reaction of the enzyme with the second substrate; 2) inhibited Ping-Pong Bi-Bi, as in 1) but in which the second substrate is a competitive inhibitor relative to the first substrate; 3) Ordered Bi-Bi, an ordered reaction involving the formation of a ternary complex of two substrates and enzyme before the catalytic step occurs; 4) Inhibited Bi-Bi, as in 3) but with the second substrate a competitive inhibitor of the first. The best fit is to 2), a ping-pong mechanism in which glycolate-2-P is competitive with glycerate-2,3-P$_2$ (Equation 6).

$$v = \frac{VAB}{BK_a(1 + \frac{B}{K_b}) + AK_a + AB}$$

where $v$ is the observed rate and $V$ the maximal velocity, $K_a$ and $K_b$ are the $K_a$ values for glycerate-2,3-P$_2$ and glycolate-2-P, respectively, and $A$ and $B$ are their concentrations. $K_{ib}$ is the inhibition constant for glycolate-2-P as a competitive inhibitor. The unusual feature is that glycolate-2-P inhibits at a lower concentration than that at which it activates, i.e. $K_{ib} < K_b$ (Table III). In addition, glycerate-2,3-P$_2$ inhibits at high concentration competitively relative to glycolate-2-P. The inhibition constant, $K_{ib}$, Table III, conforms to Equation 7.

![Fig. 1. Effect on the phosphatase rate of varying glycerate-2,3-P$_2$ (2,3-DPG) at several levels of glycolate-2-P. The incubations were at 25°C for 10 min in 0.8 ml volumes and contained 50 mM triethanolamine/Cl$^-$ buffer, pH 7.5, and 0.25 µM glycerate-2,3-[U-32P]P$_2$. The specific activity about $2 \times 10^6$ cpm/µmol was varied. Glycolate-2-P was present at 0.125 mM (C); 0.25 mM (X); 0.50 mM (Δ); 1.0 mM (□).](http://www.jbc.org/DownloadedFrom/19912327)
Kinetic Properties of Phosphoglycerate Mutase

\[ v = \frac{V_{AB}}{B \left(1 + \frac{P}{K_p}\right) + AK_1 \left(1 + \frac{A}{K_H}\right) + AB} \]  

(7)

At 25°C in 0.05 M triethanolamine/Cl\textsuperscript{-} buffer, pH 7.5, the \( k_{\text{cat}} \) is 2.78 s\textsuperscript{-1} calculated for a subunit of 30,000. The kinetic parameters have also been determined at pH 5.5 at 4°C (Table III).

Glycerate-3-P (which must rapidly form an equilibrium mixture with glycerate-2-P) inhibits competitively with glycerate-2-P, with a \( K_i \) of 2 \( \mu \text{M} \) (Fig. 2). The glycerate-2,3-P\textsubscript{2} concentration in these experiments is 7 \( \mu \text{M} \), which is 100 times the \( K_m \) of 0.065 \( \mu \text{M} \). At glycolate-2-P levels above 1 \( \mu \text{M} \), the rates decrease due to competition with glycerate-2,3-P\textsubscript{2}.

The activating effects of KCl and other salts are due to a decrease in the \( K_m \) of glycerate-2,3-P\textsubscript{2} (Fig. 3). When the KCl concentration was increased above 25 \( \mu \text{M} \), there was a decrease in the rate at high glycerate-2,3-P\textsubscript{2}, i.e., 25% inhibition with 50 \( \mu \text{M} \) KCl, indicating an increase in the \( K_m \) of glycolate-2-P, which was present at 0.6 \( \mu \text{M} \). A replot of the reciprocals of the slopes of the lines in Fig. 3 against the square of the concentration of KCl is linear which suggests that 2 eq of salt interact with the enzyme and the average apparent activation constant for KCl for each site is 17 \( \mu \text{M} \).

The effects of salts are characteristic of the anion. Identical rates were observed with equal concentrations of TES/sodium and TES/imidazole buffers at suboptimal concentrations of glycolate-2-P. Increasing the level of TES/sodium from 20 \( \mu \text{M} \) to 150 \( \mu \text{M} \) decreases the apparent \( V/K_m \) of glycerate-2,3-P\textsubscript{2} from 1.33 to 0.26 \( \mu \text{M} \), and a second TES molecule appears to interact with the enzyme at a time. The apparent activator constant for TES is 35 \( \mu \text{M} \). A replot of the slopes from double reciprocal plots of the reciprocals of the slopes against TES concentration extrapolated to zero TES gives a value of about 5 \( \mu \text{M} \) for the \( K_{\text{diss}} \) of TES.

The activating effects of KCl and other salts are due to a decrease in the \( K_m \) of glycerate-2,3-P\textsubscript{2} (Fig. 3). When the KCl concentration was increased above 25 \( \mu \text{M} \), there was a decrease in the rate at high glycerate-2,3-P\textsubscript{2}, i.e., 25% inhibition with 50 \( \mu \text{M} \) KCl, indicating an increase in the \( K_m \) of glycolate-2-P, which was present at 0.6 \( \mu \text{M} \). A replot of the reciprocals of the slopes of the lines in Fig. 3 against the square of the concentration of KCl is linear which suggests that 2 eq of salt interact with the enzyme and the average apparent activation constant for KCl for each site is 17 \( \mu \text{M} \).

The kinetic parameters for the phosphatase reaction in the presence of glycolate-2-P are given in Table III. The final pH varied by 0.10 pH unit or less. KCl was present as 0.15 M TES/sodium, pH 7.5; 7.4 \( \mu \text{M} \) glycerate-2,3-[U-\textsuperscript{32}P]P\textsubscript{2}, glycolate-2-P as given, and enzyme sufficient to use not more than 5% of the substrate. Glycerate-3-P was present as 2 \( \mu \text{M} \).

**Table III**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Parameter</th>
<th>Description</th>
<th>pH 7.5, 25°C</th>
<th>pH 5.5, 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerate-2,3-P\textsubscript{2}</td>
<td>Michaelis constant</td>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>0.065 ± 0.020 ( a )</td>
<td>0.028</td>
</tr>
<tr>
<td>Glycerate-2-P</td>
<td>Michaelis constant</td>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>479 ± 80 ( b )</td>
<td>1670</td>
</tr>
<tr>
<td>Glycerate-2,3-P\textsubscript{2}</td>
<td>Inhibitor versus glycerate-2,3-P\textsubscript{2}</td>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>135 ± 58 ( b )</td>
<td>362</td>
</tr>
<tr>
<td>Glycerate-P\textsuperscript{d}</td>
<td>Inhibitor versus glycerate-2-P</td>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>300 ( ^{c} )</td>
<td>2 ( ^{c} )</td>
</tr>
<tr>
<td>P\textsubscript{i}</td>
<td>Inhibitor versus glycerate-2-P</td>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>5000 ( ^{c} )</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Maximum velocity</td>
<td>( k_{\text{cat}} ) (s\textsuperscript{-1})</td>
<td>2.70 ( ^{b} )</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( k_{\text{cat}}/K_m ) (s\textsuperscript{-1}/M)</td>
<td>4.3 x 10\textsuperscript{5} ( ^{b} )</td>
<td>7.86 x 10\textsuperscript{5}</td>
</tr>
</tbody>
</table>

\( a \) Incubations were at 4°C for 10 min and contained 50 \( \mu \text{M} \) ammonium acetate buffer, pH 5.5. Glycerate-2,3-[U-\textsuperscript{32}P]P\textsubscript{2} was varied at three levels of glycolate-2-P, 0.5, 1, and 2 \( \mu \text{M} \).

\( b \) Conditions are given in the legend to Fig. 1. Results are from a computer analysis of five experiments.

\( c \) In 50 \( \mu \text{M} \) TES/sodium buffer, pH 7.5, containing 0.1 \( \mu \text{M} \) KCl.

\( d \) Equilibrium mixture of glycerate-2-P and glycerate-3-P.

Data from Fig. 2.
when they were tested in the same experiment. Each salt is glycerate-2,3-P₂, Cl⁻ must bind to site(s) other than the site(s) observed among Na⁺, K⁺, and NH₄⁺ salts of the same anion 818 and 943, respectively, and KC₁ and NH₄Cl change it to TES/sodium buffer for which the observed 

Effects of salts on the $K_m$ of glycerate-2,3-P₂ in the phosphatase reaction

All incubations were at pH 7.5 and contained 0.5 mM glycolate-2-P and 10 mM triethanolamine/Cl⁻ buffer.

<table>
<thead>
<tr>
<th>Salt composition</th>
<th>Apparent parameters</th>
<th>$V/K_m$</th>
<th>$K_m$, $2\text{-}3\text{-DPG}$, $\mu$M</th>
<th>$V$, $\mu$M/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>TES/sodium, 50 mM (8)</td>
<td>$0.527 \pm 0.077$</td>
<td>$98.3 \pm 9.1$</td>
<td>$189 \pm 26$</td>
<td></td>
</tr>
<tr>
<td>TES/sodium, 50 mM + KAc, 50 mM</td>
<td>$0.088$</td>
<td>$48$</td>
<td>$818$</td>
<td></td>
</tr>
<tr>
<td>TES/sodium, 50 mM + NH₄Ac, 50 mM</td>
<td>$0.062$</td>
<td>$39$</td>
<td>$943$</td>
<td></td>
</tr>
<tr>
<td>TES/sodium, 50 mM + KCl, 50 mM</td>
<td>$0.0599$</td>
<td>$53.4$</td>
<td>$1338^d$</td>
<td></td>
</tr>
<tr>
<td>TES/sodium, 50 mM + NH₄Cl, 50 mM</td>
<td>$0.0336$</td>
<td>$36.5$</td>
<td>$1629^d$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 2,3-DPG, glycerate-2,3-P₂.

$^b$ Micromoles/min/µmol of subunits.

$^c$ Number of experiments.

$^d$ Calculated using $V$ corrected to eliminate inhibition of glycolate-2-P by salt.

TES/sodium buffer for which the observed $V/K_m$ (apparent) is 189, potassium acetate and NH₄-acetate change the ratio to 818 and 943, respectively, and KCl and NH₄Cl change it to 1338 and 1629 (Table IV). No significant differences were observed among Na⁺, K⁺, and NH₄⁺ salts of the same anion when they were tested in the same experiment. Each salt is also inhibitory relative to glycolate-2-P as shown by the effects of higher levels of salts on the ordinate intercepts in Figs. 3 and 4.

When the KCl concentration is increased, up to 0.4 M, in the presence of 0.5 mM glycolate-2-P and variable glycerate-2,3-P₂, the lines of double reciprocal plots reach a constant slope that indicates a limiting low $K_m$ (Fig. 5) of about 0.04 µM. A replot of the intercepts on the ordinate against the square of the KCl concentrations is linear indicating an average dissociation constant of 61 mM for KCl as an inhibitor relative to glycolate-2-P. In order to decrease the $K_m$ of glycerate-2,3-P₂, Cl⁻ must bind to site(s) other than the substrate site. If it binds to the substrate site as well, Cl⁻ should exhibit competitive inhibition as the salt concentration is increased. These observations are consistent with the postulate that salts react at the binding site for the second substrate (glycolate-2-P) and are excluded from a separate glycerate-2,3-P₂ site. This would explain the ability to bind two Cl⁻ ions or one P₃ (or one of each to accelerate the phosphatase rate); TES is much larger than Cl⁻ and only one molecule gets into the site. Another way to achieve this specificity would be if salts bind to the substrate site on the second subunit or to an allosteric site with some substrate-like specificity.

**Phosphoglycerate Mutase Reaction**

**Absolute Requirement for Glycerate-2,3-P₂**—The phosphoglycerate mutase reaction results in the transfer of a phosphoribyl group between the 2- and 3-positions of D-phosphoglycerate. The enzyme can be phosphorylated by the substrate, glycerate-2,3-P₂, on a histidine residue (3, 4), and the phospho-enzyme is believed to function in the catalytic reaction (6). The phosphohistidine bond is not stable during the isolation of the enzyme. Although early workers found little or no phosphatase in purified preparations of phosphoglycerate mutase, they reported low residual mutase activity in the absence of added glycerate-2,3-P₂ (see Ray and Peck (32)). Since this result is essential to our understanding of the enzymatic mechanism, we have reinvestigated and found that using phosphoglycerates purified on ion exchange columns as substrates we can detect no mutase activity in the absence of added glycerate-2,3-P₂. The addition of glycerate-2,3-P₂ in amounts less than equimolar with enzyme gives measurable activity.

**Kinetic Studies**—When glycerate-2-P is varied in the presence of a wide range of levels of glycerate-2,3-P₂ including values well below the $K_m$, the pattern of the double reciprocal plots is similar to that observed for the phosphatase reaction shown in Fig. 1 and is consistent with an inhibited ping-pong mechanism. The data in Fig. 6A were analyzed graphically according to Equation 6 where $B$ = glycerate-2-P and the values obtained for the kinetic parameters are shown in Table V. The lines in Fig. 6B are drawn with these values, using the same equation, and serve to illustrate better the features of this mechanism and the fit of the data from this experiment. Also included in Fig. 6B are data from an additional experiment to extend the concentration of glycerate-2,3-P₂ to 1 µM. Marked inhibition was observed with high glycerate-2-P (5 to 20 µM) in the presence of low glycerate-2,3-P₂ (0.01 µM). With glycerate-2-P below saturation (2.5 µM), no inhibition by glycerate-2,3-P₂ was observed at levels up to 100 µM (>1000 times the $K_m$ of glycerate-2,3-P₂).

**FIG. 5. Effects of high salt on the phosphatase reaction.** The incubations contained 0.4-ml volumes, 50 mM TES/sodium buffer, pH 7.5, 0.5 mM glycolate-2-P, and enzyme to utilize about 10 to 20% of the substrate. Glycerate-2,3-[U-³²P]P₂ and KCl were varied as indicated; KCl: 0 M (a), 0.05 M (X), 0.2 M (o), 0.4 M (C). The (+) indicates data from another experiment with 0.2 or 0.4 M KCl, 2,3-DPG, glycerate-2,3-P₂.
**Kinetic Properties of Phosphoglycerate Mutase**

The \( K_m \) of glycerate-3-P was estimated in two ways. It should be possible to calculate a value from the Haldane relationship (Equation 8) (33), where \( V_i \) and \( V_v \) refer to the maximal rates in the forward and reverse directions. Ray and Roscelli (34) showed that this approach is applicable when studying the mechanistically similar phosphoglucomutase reaction.

\[
K_{eq} = \frac{V_i K_m(\text{glycerate-3-P})}{V_v K_m(\text{glycerate-2-P})}
\]  

(8)

The equilibrium constant for the phosphoglycerate mutase reaction \( (K_{eq} = \text{glycerate-3-P}/\text{glycerate-2-P}) \) had been reported to be 5 to 6 (35). More recently, Britton et al. (36) have reported a value of 11.92. In the course of this work, a value of 12.7 at pH 7.5 and 25°C in the absence of Mg\(^{2+}\) was obtained. The unfavorable equilibrium accounts for the rapid onset of inhibition when glycerate-3-P is substrate (35). The \( K_m \) of glycerate-3-P is then 12 times that of mal velocity in both directions (6). Using 12 as the equilibrium constant, the \( K_m \) of glycerate-3-P is then 12 times that of glycerate-2-P or 168 \( \mu \)M. We have also measured the \( K_m \) of glycerate-3-P directly using the enolase coupled assay and have obtained a value of 250 \( \mu \)M. This assay system is not really satisfactory for these measurements as has been noted (32). With saturating glycerate-2,3-P\(_2\) and keeping the extent of reaction <10%, the observed rates are low and not constant with time.

**Effects of Salts**—As reported by Grisolia and Cleland (11), salts affect the rate of the mutase reaction, KCl is a competitive inhibitor with respect to glycerate-2-P (Fig. 7). A replot of the slopes against the square of the salt concentration is linear, implicating interaction with 2 eq of salt per functional unit. The apparent \( K_i \) is 60 mM which agrees with the \( K_m \) of Cl\(^{-}\) relative to glycolate 2 P in the phosphatase reaction (Fig. 5). The effect of increasing KCl on the \( K_m \) of glycerate-2,3-P\(_2\), with glycerate-2-P at a nonsaturating level of 2 \( \mu \)M, is shown in Fig. 8. When the KCl concentration is 50 mM or less, the effect on the slope is small indicating that the decreased rate is due largely to the increase in the \( K_m \) of glycerate-2,3-P\(_2\). At 75 or 100 mM KCl, there is a marked increase in the slope as well (Fig. 8) which indicates competition with glycerate-2,3-P\(_2\).

There are isotopic data which show that free glycerate-2,3-P\(_2\) is not involved in each turnover of the enzyme (19). Salt accelerates the dissociation of glycerate-2,3-P\(_2\) from the enzyme, marked effects occurring only with salt concentrations above 0.1 M; at 0.5 to 1 ionic strength the rate of dissociation of glycerate-2,3-P\(_2\) from the enzyme can exceed the rate of the overall reaction (19).

**Inhibition by Phosphate and Glycolate-2-P**—Inorganic phosphate and glycolate-2-P are competitive inhibitors relative to both glycerate-2-P and glycerate-2,3-P\(_2\). The \( K_i \) for P\(_i\) is about 3.5 mM with respect to each substrate. Glycolate-2-P has a \( K_i \) of 150 \( \mu \)M relative to glycerate-2,3-P\(_2\) and 1.4 mM relative to glycerate-2-P. The inhibitor constant for glycolate-2-P relative to glycerate-2,3-P\(_2\) in the phosphatase reaction is 135 \( \mu \)M which is very close to the \( K_i \) found for the mutase reaction.

**Bisphosphoglycerate Synthase (Mutase) Activity**

Alpers and co-workers (17) have shown that glycerate-1,3-P\(_2\) can stimulate the mutase activity in the absence of glycerate-2,3-P\(_2\) and also produce glycerate-2,3-P\(_2\), de novo. In agreement with these observations, we have demonstrated the phosphorylation of the enzyme by glycerate-1,3-P\(_2\) (6). The dependence of the overall synthase reaction on added glycerate-3-P is shown in Fig. 9. At pH 7.5, 25°C, in 10-min assays with glycerate-3-P saturating, the observed \( K_m \) value for glycerate-1,3-P\(_2\) is 10 to 20 \( \mu \)M, and a \( k_{cat} \) of 0.4 s\(^{-1}\) was calculated. These values are probably in error since it was observed that the rate deviated from linearity even when 5% of the substrate had been utilized, probably because of inhibition by the glycerate-2,3-P\(_2\) produced. The inhibition would

---

**Table V**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Parameter</th>
<th>Designation</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerate-2,3-P</td>
<td>Michaelis constant</td>
<td>( K_m ) (( \mu )M)</td>
<td>0.069</td>
</tr>
<tr>
<td>Glycerate-2-P</td>
<td>Michaelis constant</td>
<td>( K_m ) (( \mu )M)</td>
<td>14</td>
</tr>
<tr>
<td>Glycerate-3-P</td>
<td>Michaelis constant (reverse reaction)</td>
<td>( K_m ) (( \mu )M)</td>
<td>168(^b)</td>
</tr>
<tr>
<td>Glycerate-2-P</td>
<td>Inhibitor versus glycerate-2,3-P(_2)</td>
<td>( K_i ) (( \mu )M)</td>
<td>3.9</td>
</tr>
<tr>
<td>Glycolate-2-P</td>
<td>Inhibitor versus glycerate-2-P</td>
<td>( K_i ) (( \mu )M)</td>
<td>1400</td>
</tr>
<tr>
<td>Glycolate-2-P</td>
<td>Inhibitor versus glycerate-2,3-P(_2)</td>
<td>( K_i ) (( \mu )M)</td>
<td>150</td>
</tr>
<tr>
<td>( P_i )</td>
<td>Inhibitor versus glycerate-2-P</td>
<td>( K_i ) (( \mu )M)</td>
<td>3500</td>
</tr>
<tr>
<td>( P_i )</td>
<td>Inhibitor versus glycerate-2,3-P(_2)</td>
<td>( k_{cat} ) (s(^{-1}))</td>
<td>1197</td>
</tr>
<tr>
<td>( k_{cat} / K_i ) (s(^{-1})/M)</td>
<td>( 1.72 \times 10^4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Calculated from the Haldane relationship.

\(^b\) Direct determination.
incubations and enzyme sufficient to use up to 15% of the substrate. [2T]Glycerate-2-P and KCl were varied: KCl, none (○), 50 mM (×), and 100 mM (△).

Fig. 7. Effect of KCl on the $K_m$ of glyceraldehyde-2,3-P$_2$ (2-PGA). The incubations were at 25°C for 10 min in 0.1- or 0.2-ml volumes and contained: 50 mM TES/sodium buffer, pH 7.5, 2 μM glyceraldehyde-2,3-P$_2$, and enzyme sufficient to use up to 15% of the substrate. [2T]Glycerate-2-P and KCl were varied: KCl, none (○), 50 mM (×), and 100 mM (△).

Fig. 8 (left). Effect of KCl on the $K_m$ of glyceraldehyde-2,3-P$_2$ (2,3-DPG) (mutase). The incubations contained 50 mM TES/sodium buffer, pH 7.5, 2 μM glyceraldehyde-2,3-P$_2$, and enzyme sufficient to use up to 15% of the substrate. Glyceraldehyde-2,3-P$_2$ and KCl were varied: no KCl (○); 50 mM (×); 100 mM (△).

Fig. 9 (right). Stimulation by glyceraldehyde-3-P (3-PGA) of the utilization of glyceraldehyde-1,3-P$_2$ to form glyceraldehyde-2,3-P$_2$. The incubations were for 10 min at 25°C and contained in 0.2-ml volume: 10 mM TES/sodium buffer, pH 7.5; 5 mM β-mercaptoethanol; 2.5 μM glyceraldehyde-1,3-[1-14C]P$_2$, and enzyme (5 × 10$^{-6}$ μmol). Glyceraldehyde-3-P$_2$ was added as indicated. The extrapolation of the data points indicates that the glyceraldehyde-1,5-P$_2$ solution contained 0.8 μM glyceraldehyde-3-P$_2$.

give too high an apparent $K_m$ for glyceraldehyde-1,3-P$_2$ and too low an observed maximal velocity. The high affinity of glyceraldehyde-1,3-P$_2$ for the enzyme was observed earlier. The rate of phosphorylation of the enzyme was not dependent on the level of glyceraldehyde-1,3-P$_2$ even at 0.5 μM, which represented a 4-fold excess of substrate over enzyme ($K_{bind} < 10^{-8}$ M) (6). The measured rate of phosphorylation was 1.57 s$^{-1}$.

**Discussion**

Reaction schemes for the three activities of phosphoglycerate mutase are presented (Fig. 10). The proposed mechanism for the mutase is ping-pong as suggested by Grisolia and Cleland (11). As written, no distinction is made between E-P formed with glycerate-2-P or glycerate-3-P. This is consistent with the work of Britton et al. (37, 38) who could detect no rate-limiting isomerizations in the reactions of yeast or muscle phosphoglycerate mutase. Winn et al. (8) have suggested that there are 2 histidine residues at the active site of yeast phosphoglycerate mutase that may participate by being phosphorylated alternately by a specific phosphoryl group of glycerate-2,3-P$_2$. If so, the transfer of the phosphoryl group between the two forms of E-P is very rapid. The phosphatase reaction also has a modified ping-pong mechanism. It has been simplified to include the minimal number of rapid steps (i.e., steps that occur in the mutase reaction). Rate equations were derived manually for the mutase and phosphatase reactions using the procedure of Indge and Childs (39) (Table VI). Inhibition by the second substrate was included in the derivations but not inhibition by glycerate-2,3-P$_2$ which was not observed up to 100 μM substrate, 1000 times the $K_m$ under the conditions studied. For initial conditions, both rate equations are in the form of Equation 3, as required by the kinetic data. The expressions for the kinetic parameters will be used to try to understand the effects of salts and particularly the observation that the apparent $K_m$ of glyceraldehyde-2,3-P$_2$ decreases with salt in the phosphatase reaction and increases under the same conditions in the mutase reaction.

We have shown that phosphatase activity is not observed in the absence of glycolate-2-P or another activator (Table 1). The activators are all related in structure to the mutase substrates, the monophosphoglycerates; in order of increasing effectiveness, P$^i$ < pyrophosphate (27, 28), <glycolate-2-P (16, 22, 29) and phosphohydroxypyruvate (16, 30). With glycolate-2-P as activator, the phosphatase rate can reach 1/400 ($k_{cat}$ 2.78 s$^{-1}$) of the mutase rate ($k_{cat}$ 1197 s$^{-1}$). Rate constants that occur in both reactions must be fast enough to allow the mutase role and so cannot be rate-limiting for the phosphatase. In rapid mixing and quenching studies (6), the rate of phosphoryl transfer from the enzyme in the presence of glycolate-2-P was determined ($k_{cat}$ 3.59 s$^{-1}$), and it is close to the observed $V$. In the $V$ expression (Table VI), the denominator factor, $Z$, is dominated by $k_{cat}k_2$, both of which rate constants are in the mutase rate (the other terms are factors of $k_b$ and must be much smaller). Therefore, $V$ is approximately equal to $k_b$ as has been observed. The maximal velocity is not affected by salt (Figs. 3 to 5) and therefore $k_b$ does not change with salt concentration. To evaluate the $K_m$ of glyceraldehyde-2,3-P$_2$, isotopic evidence indicates that in low salt $k_{cat}$, the off rate of glyceraldehyde-2,3-P$_2$ is low relative to $k_2$ and $k_3$ (19). Then, with the same logic that was used to simplify the expression for $V$, the $K_m$ of glyceraldehyde-2,3-P$_2$ in low salt is $k_{cat}/k_b$. The effects of salts are due then to an increase in $k_b$, the on rate for glyceraldehyde-2,3-P$_2$, which causes a decrease in the $K_m$ of glyceraldehyde-2,3-P$_2$.

It is possible to evaluate $k_1$ from the expression for the $K_m$ of glyceraldehyde-2,3-P$_2$ in the phosphatase reaction, $K_m = k_b/k_1$, and the value for $k_b$ obtained in earlier quench studies (6). The limiting $K_m$ in the absence of salt was calculated to be 5 μM or 5 × 10$^{-9}$ M and therefore in the absence of salt $k_b = 7.2$ × 10$^{-10}$ M$^{-1}$ s$^{-1}$. From the change in the apparent $K_m$ of glyceraldehyde-2,3-P$_2$ with KCl (Fig. 3), the variation of $k_b$ with KCl is related to the square of the Cl$^-$ concentration with an apparent "$K_0"$ value of 17 mM. The $K_m$ decreased to 0.04 μM in the presence of 0.1 or 0.4 M KCl and 50 mM TES buffer, pH 7.5 (Fig. 5), indicating a 100-fold increase in $k_b$ to nearly 10$^{-7}$ M$^{-1}$ s$^{-1}$. The constant low value of the $K_m$ of glyceraldehyde-2,3-P$_2$ when KCl is varied from 0.1 to 0.4 M (Fig. 5) indicates that $k_1$ has remained low relative to $k_2$ and $k_3$ above 0.5 ionic strength the $K_m$ would be expected to increase.

In order to decrease the $K_m$ of glyceraldehyde-2,3-P$_2$, Cl$^-$ must bind to site(s) other than the substrate site. If it binds to the substrate site as well, Cl$^-$ should become a competitive inhibitor as the salt concentration is increased. However, as the salt is increased up to 0.4 M, the $K_m$ of glyceraldehyde-2,3-P$_2$ reaches a low constant value as indicated by the constant slopes of the...
The kinetic parameters obtained in 50 mM TES buffer containing 0.1 M KCl, pH 7.5, 25°C are: $k_{\text{cat}}$, 1197 s$^{-1}$ (6) and $K_m$ of glycerate-2,3-P$_2$, $6.9 \times 10^{-4}$ M so $V/K_m$ is $1.17 \times 10^{10}$ M$^{-1}$ s$^{-1}$. This is in the range for a diffusion-controlled reaction. For such a reaction, the off rate must be small relative to the rate of the catalytic step so that Equation 9 simplifies to $V/K = k_{\text{cat}}/k_{\text{off}}$. The discrepancies in the form of the equation and the value obtained for $V/K_m$ arise because in the mutase reaction the combination of the enzyme with substrate does not occur with each catalytic event. At ionic strength much less than 0.5, free glycerate-2,3-P$_2$ participates infrequently relative to the rate of the overall reaction (19). At higher salt levels, $k_{-1}$, the off rate of the substrate, becomes more important, the $K_m$ of glycerate-2,3-P$_2$ increases, and $V/K_m$ will decrease.

The observations that salts react with the mutase at a site other than that at which glycerate-2,3-P$_2$ phosphorylates the enzyme and that the same salts are competitive with the

> lines in double reciprocal plots (Fig. 5). As shown by the intercepts of the lines, the salt increasingly inhibits relative to glycolate-2-P. The kinetic effects of Cl$^-$ on both the $K_m$ of glycerate-2,3-P$_2$ and that of glycolate-2-P are related to the square of the Cl$^-$ concentration.

The kinetic parameters of the mutase reaction are also sensitive to salt concentration. KCl inhibition is competitive with glycerate-2-P, and the $K_m$ of glycerate-2-P increases with the square of the concentration of KCl (Fig. 7). There is an apparently nonlinear increase in the $K_m$ of glycerate-2,3-P$_2$ with increasing KCl concentration (Fig. 8). The $K_m$ expression (Table VI) contains rate constants included in the $V$ (independent of salt, Figs. 7 and 8) plus the factor $k_{-1}/k_1$, the on-off rate of glycerate-2,3-P$_2$ which is affected by salt. Salt could increase the $K_m$ of glycerate-2,3-P$_2$ as a competitive inhibitor that binds to the substrate site, or by an increase in $k_{-1}/k_1$, or both. Since the results from the phosphatase study (Fig. 5) indicate that salt does not react at the glycerate-2,3-P$_2$ binding site, it appears likely that the salt effect on the $K_m$ can be attributed solely to an increase in $k_{-1}/k_1$.

The value of $V/K_m$ for glycerate-2,3-P$_2$ in the mutase reaction (Table VI) does not conform to the usual expression (40, 41).
**Kinetic Properties of Phosphoglycerate Mutase**

**TABLE VI**

Kinetic equations for phosphoglycerate mutase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phosphatase Activity</th>
<th>Mutase Activity (2-PGA → 3-PGA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>v</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \frac{a}{b} \frac{AC}{[E]_t} )</td>
<td>( \frac{p}{q} \frac{AB}{[E]_t} )</td>
</tr>
<tr>
<td></td>
<td>( \frac{c}{b} \frac{C}{(1 + \frac{C}{c/d})} + \frac{e}{b} \frac{A + AC}{q} )</td>
<td>( \frac{pB}{q} \left(1 + \frac{B}{r/t}\right) + \frac{e}{q} \frac{A + AB}{t} )</td>
</tr>
<tr>
<td>V</td>
<td>( \frac{k_2k_3k_9}{Z} ) = ( k_9 )</td>
<td>( \frac{k_2k_4k_5}{Y} )</td>
</tr>
<tr>
<td></td>
<td>( \frac{k_9(k_{-1}k_{-2} - 1 + k_{-4}k_{-3} + k_{-2}k_{-3})}{k_1 Z} ) = ( k_9 )</td>
<td>( \frac{k_9k_{-1}k_{-4}k_{-3}}{k_1 Y} )</td>
</tr>
<tr>
<td>( K_m ) A</td>
<td>( \frac{k_9(k_{-1}k_{-2} + k_{-3}k_{-4} + k_{-2}k_{-3})}{k_1 Z} ) = ( k_9 )</td>
<td>( \frac{k_9k_{-1}k_{-4}k_{-3}}{k_1 Y} )</td>
</tr>
<tr>
<td>( K_m ) B</td>
<td>( \frac{k_9(k_{-1}k_{-2} + k_{-3}k_{-4} + k_{-2}k_{-3})}{k_1 Z} ) = ( k_9 )</td>
<td>( \frac{k_9k_{-1}k_{-4}k_{-3}}{k_1 Y} )</td>
</tr>
<tr>
<td>( K_m ) C</td>
<td>( \frac{k_9(k_{-1}k_{-2} + k_{-3}k_{-4} + k_{-2}k_{-3})}{k_1 Z} ) = ( k_9 )</td>
<td>( \frac{k_9k_{-1}k_{-4}k_{-3}}{k_1 Y} )</td>
</tr>
<tr>
<td>( K_l ) C</td>
<td>( \frac{k_{-10}/k_{10}}{k_9} )</td>
<td>( \frac{k_{-6}/k_{6}}{k_9} )</td>
</tr>
<tr>
<td>( K_i ) B</td>
<td>( \frac{k_{-10}/k_{10}}{k_9} )</td>
<td>( \frac{k_{-6}/k_{6}}{k_9} )</td>
</tr>
<tr>
<td>( V/K_a )</td>
<td>( \frac{k_1 \cdot \frac{k_2k_3}{k_2 + k_3}}{k_2 + k_3} )</td>
<td>( \frac{k_1 \cdot \frac{k_2k_3}{k_2 + k_3}}{k_2 + k_3} )</td>
</tr>
<tr>
<td>(second</td>
<td>( \frac{k_1 \cdot \frac{k_2k_3}{k_2 + k_3}}{k_2 + k_3} )</td>
<td>( \frac{k_1 \cdot \frac{k_2k_3}{k_2 + k_3}}{k_2 + k_3} )</td>
</tr>
<tr>
<td>substrate)</td>
<td>( \frac{k_8 \cdot k_9}{k_8 + k_9} )</td>
<td>( \frac{k_8 \cdot k_9}{k_8 + k_9} )</td>
</tr>
<tr>
<td>( \frac{E_9}{t} )</td>
<td>( \frac{[E]_t}{k_8 + k_9} )</td>
<td>( \frac{[E]_t}{k_8 + k_9} )</td>
</tr>
</tbody>
</table>

\( [E]_t \) = total enzyme concentration

A = 2,3-DPG

B = 2-PGA

Y = \( \frac{k_9k_{-4} + k_9k_{-5} + k_9k_{-6}}{k_9k_{-4} + k_9k_{-5} + k_9k_{-6}} \)

p = \( k_1k_2k_3k_4k_5 \)

q = \( k_1k_2k_3k_4k_5 + k_2k_3k_4k_5 \)

r = \( k_1k_2k_3k_4k_5 \)

s = \( k_1k_2k_3k_4k_5 \)

A = 2,3-DPG

B = 2-PGA

Y = \( \frac{k_9k_{-4} + k_9k_{-5} + k_9k_{-6}}{k_9k_{-4} + k_9k_{-5} + k_9k_{-6}} \)

p = \( k_1k_2k_3k_4k_5 \)

q = \( k_1k_2k_3k_4k_5 + k_2k_3k_4k_5 \)

r = \( k_1k_2k_3k_4k_5 \)

s = \( k_1k_2k_3k_4k_5 \)
second substrates glycerate-2-P and glycolate-2-P, lead us to postulate that there are two substrate-binding sites at each active site of the enzyme. We propose that one site is specific for glycerate-2-P and the other for glycerate-3-P and that glycerate-2,3-P₂ can bind to either site and phosphorylate the same histidine residue. Then, as indicated in the scheme (Fig. 10), phosphoenzyme can donate the phosphoryl group to added phosphoglycerate to reform glycerate-2,3-P₂. At low or moderate salt levels, the glycerate-2,3-P₂ thus formed must be able to reorient itself before it is released from the enzyme in such a way that the alternate phosphoryl bond will be donated to the enzyme. When salts bind to one of the sites on the nonphosphorylated enzyme, a conformational change occurs that allows an increase in the rate at which glycerate-2,3-P₂ goes on and off the alternate site. The possibility of two sites for binding the substrates of this enzyme was suggested previously to explain some observations on the rate of phosphorylation by glycerate-2,3-P₂ (6). The participation of 2 Cl⁻ in each interaction is reminiscent of the synergistic effects of Cl⁻ and Pᵢ in the activation of the glycerate 2,3-P₂ phosphatase activity of bisphosphoglycerate synthase in which case the combination was interpreted to be a poor analog of the substrate, phosphoglycerate (22). Also, for the synthase reaction catalyzed by bisphosphoglycerate synthase, the presence of glycerate 2 P increased the maximal velocity of phosphorylation of glycerate-3-P and decreased its Kₘ (25).

The bisphosphoglycerate synthase activity of phosphoglycerate mutase allows the possibility for the mutase to function in a cell that lacks a separate enzyme to synthesize glycerate-2,3-P₂. Glycerate-1,3-P₂ is produced during glycolysis by glyceraldehyde-3-P dehydrogenase, and it can serve as the phosphoryl donor for phosphoglycerate mutase with a high affinity of ≥7 × 10⁻⁹ M (6). The rate of phosphorylation by bound glycerate-1,3-P₂ is slow and probably rate-limiting (kₐₚ 1.57 s⁻¹) in contrast to that of glycerate-2,3-P₂ (≥100 s⁻¹) (6). We have shown that the synthase reaction requires added glycerate-3-P (Fig. 9). This could be due to the rapid release of the first product or is compatible with a separate binding site for the acceptor molecule. Other properties of phosphoglycerate mutase maximize the usefulness of an interaction with glycerate-1,3-P₂; the phosphorylation event is not required with each turnover of the enzyme; when the phosphoryl group is released it would be in the form of glycerate-2,3-P₂ which is then phosphorylated on a histidine residue by either glycerate-1,3-P₂ or glycerate-2,3-P₂ (18). The glycerate-2,3-P₂ produced from glycerate-1,3-P₂ is released at each turnover of the enzyme. Therefore, at physiological salt concentration (~0.1 M) the synthase has properties induced into phosphoglycerate mutase by unphysiologically high levels of salt. Comparing the activities of synthase from horse red blood cells to phosphoglycerate mutase (chicken breast muscle), the result is an enhancement of the synthase activity (kₚ 2.57 s⁻¹) (6). The distribution of the synthase is not well established, and it is possible that phosphoglycerate mutase may actually use glycerate-1,3-P₂ for phosphorylation in some cells.

Acknowledgments—We wish to thank Dr. Jack London, Department of Radiology, Hospital of the University of Pennsylvania, for the computer analysis of the kinetics of the phosphatase reaction. We appreciate the excellent technical assistance of Janine Corbett.

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Z B Rose and S Dube


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