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HUMAN ERYTHROCYTE PHOSPHOGLYCERATE MUTASE: EVIDENCE FOR NORMAL CATALYSIS IN THE ABSENCE OF ADDED 2,3-BISPHOSPHO-D-GLYCERATE

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Summary: Kinetic analyses indicate that human erythrocyte phosphoglycerate mutase catalyzes the normal, reversible isomerization of D-glycerate-3-P and D-glycerate-2-P in the absence of added D-glycerate-2,3-P_2. The reaction is impeded, however, by a potent inhibitor which occurs as a natural component of commercial D-glycerate-3-P. Inhibition may be overcome through substrate purification or by adding D-glycerate-2,3-P_2 to the reaction medium containing the contaminant. In surmounting the inhibition, bisphosphoglycerate performs as a non-essential activator and not as a cofactor. The latter concept is corroborated by the observation that D-glycerate-2,3-P_2 has absolutely no influence on mutase catalysis conducted in the presence of pure substrate. The data presented here and elsewhere, however, suggest that the red cell enzyme is readily phosphorylated by mono- as well as bisphosphoglycerate. Additional findings show that at concentrations in excess of 3mM, D-glycerate-3-P accelerates phosphoglycerate mutase catalysis in the absence of cofactor, suggesting that the mutase molecule possesses a normal catalytic site and an allosteric activator site.

Phosphoglycerate mutase (EC 2.7.5.3) catalyzes the interconversion of D-glycerate-3-P (3-PGA) and D-glycerate-2-P (2-PGA)(1). Following the demonstration by Sutherland *et al.* (2,3) that D-glycerate-2,3-P_2 (2,3-DPG) dramatically accelerates mutase activity, 2,3-DPG has been implicated as a required cofactor during catalysis by phosphoglycerate mutase found in yeast and the tissues of higher animals (4). In contrast, phosphoglycerate mutase obtained from wheat germ, rice germ, and other plant sources manifests no cofactor dependence whatsoever (5-7).

Studies by Rose and co-workers (8-10) have demonstrated that ^{32}P-labeled glycerate-2,3-P_2 is capable of phosphorylating a histidyl residue of skeletal muscle and yeast phosphoglycerate mutase. In each case, the covalently bound ^{32}P is readily transferred to either 2-PGA or 3-PGA, but undergoes rapid hydrolysis in the presence of the abortive substrate, glycolate-2-P. Additional results obtained by rapid quench techniques indicate that a phosphoryl enzyme is

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a normal, kinetically competent intermediate in the 2,3-DPG-dependent mutase reaction sequence (11). Recently, Knowles and co-workers (7,12) have presented evidence that wheat germ phosphoglycerate mutase also catalyzes a pathway involving a phosphoryl-enzyme intermediate.

Isoelectric focusing experiments indicate that the phosphoglycerate mutase associated with circulating human erythrocytes is fully phosphorylated because of exposure to high intracellular concentrations of 2,3-DPG (13-15); however, enzyme dephosphorylation (as manifest by a cathodic shift in electrofocusing pattern) is rapidly effected in the presence of glycolate-2-P, but not in the presence of the normal acceptor, D-glycerate-3-P (14-16). The latter finding is apparently discordant with the tenets established by Rose et al. (8,11) for phosphoglycerate mutase phosphoryl transfer to suitable acceptors.

In an effort to decipher the above discrepancy, we considered reaction schemes which could account for the concomitant transfer and retention of a phosphoryl group by E-P. Two such schemes are as follows:

$$EP + 3\text{-PGA} \rightleftharpoons EP\ (3\text{-PGA}) \rightleftharpoons E(2,3\text{-DPG}) \rightleftharpoons EP(2\text{-PGA}) \rightleftharpoons EP + 2\text{-PGA} \qquad (1)$$

$$\begin{aligned} &EP + 3\text{-PGA} \rightleftharpoons EP(3\text{-PGA}) \rightleftharpoons E(2,3\text{-DPG}) \rightleftharpoons E + 2,3\text{-DPG} \\ &E + 3\text{-PGA} \rightleftharpoons E(3\text{-PGA}) \rightleftharpoons EP(\text{Glycerate}) \rightleftharpoons EP + \text{Glycerate} \end{aligned} \qquad (2)$$

Scheme 1 represents the classical reaction pathway for 2,3-DPG-dependent mutases. The second scheme is a variation of the first and shows that after 2,3-DPG is formed, it is released intact from the enzyme surface. The resultant phosphate-free phosphoglycerate mutase is then rephosphorylated by 3-PGA, with the subsequent liberation of D-glycerate.

The possibility that human erythrocyte phosphoglycerate mutase might be phosphorylated by either 3-PGA or 2,3-DPG led us to perform the steady-state kinetic analyses described in this report.

MATERIALS AND METHODS

Materials: Anion exchange resin AG-1-x8 (200-400 mesh) was obtained from Bio-Rad Laboratories. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

(HEPES), the pentacyclohexylammonium salt of D-glycerate-2,3-P_2 (Grade I), and the disodium salt of D-glycerate-3-P (Grade I) were purchased from Sigma. Commercially available enzymes were procured from Boehringer-Mannheim. All solutions were prepared with reagent-grade chemicals and glass-distilled water.

Red Cell Phosphoglycerate Mutase Preparation: Phosphoglycerate mutase from outdated human erythrocytes was purified through Step 7 of the method of Sheibley and Hass (17). At this stage of purification, the enzyme contained no bisphosphoglycerate synthase activity and manifested no evidence of being phosphorylated as indicated by isoelectric focusing (13,15). Enzyme concentrations were ascertained employing an absorbance index (1.0 mg/ml at 280 nm) of 1.56/cm (18).

Purification of D-glycerate-3-P: Commercial D-glycerate-3-P (50 µmoles) was purified on a 1.5 x 14 cm AG-1-x8 (200-400 mesh) formate column as described by Bartlett (19). After removal of ammonium formate by fractional sublimation at 90°C, the 3-PGA was adjusted to pH 7.0 with NaOH and subsequently treated with charcoal to remove ultraviolet absorbing material. 3-PGA concentrations were determined by the methods of Bartlett (20), and Czok and Eckert (21).

Kinetic Analyses: The enolase-coupled assay of Grisolia (22) was used for conducting kinetic analyses. Assays were performed at pH 7.0 and 25°C in a volume of 1.0 ml containing 20 µmoles HEPES, 2.5 µmoles $MgSO_4$, 0.8 U enolase, phosphoglycerate mutase, 3-PGA, and 2,3-DPG. In many cases, 2,3-DPG was omitted from the reaction mixture. Mutase concentrations were adjusted to yield linear initial reaction velocities. The amount of mutase used was estimated from the specific activity of 515 U/mg reported for the purified red cell enzyme (18). The conversion of 3-PGA to 2-PGA was related to the moles of P-enolpyruvate formed, using the extinction coefficient, $\varepsilon_{240} = 1.1 \times 10^{-3}\ M^{-1}cm^{-1}$ (23). Initial reaction velocities were expressed as the µmoles of product released/min/mg enzyme.

RESULTS

Several kinetic studies were performed in an effort to ascertain the capability of red cell phosphoglycerate mutase to catalyze the conversion of 3-PGA to 2-PGA in the absence of added 2,3-DPG. Throughout all of the studies reported here, dephosphoenzyme, as indicated by electrofocusing methods (13,15), was used, eliminating the possibility of forming 2,3-DPG *via* the following reaction sequence.

$$EP + 3\text{-PGA} \longleftrightarrow EP(3\text{-PGA}) \longleftrightarrow E(2,3\text{-DPG})$$

Fig. 1 illustrates the family of double reciprocal plots obtained by varying the concentration of 3-PGA at different stages of substrate purity. As indicated, mutase catalysis indeed occurs without the addition of 2,3-DPG to the reaction medium. Equally phenomenal, however, is the extremely potent inhibition of the initial reaction velocity observed when the variable substrate is contaminated with high levels of commercial 3-PGA. Thus 100% commercial 3-PGA (solid circles) yields a V_{max} which is approximately 1.5 orders of magnitude less than that obtained with purified 3-PGA (open triangles).

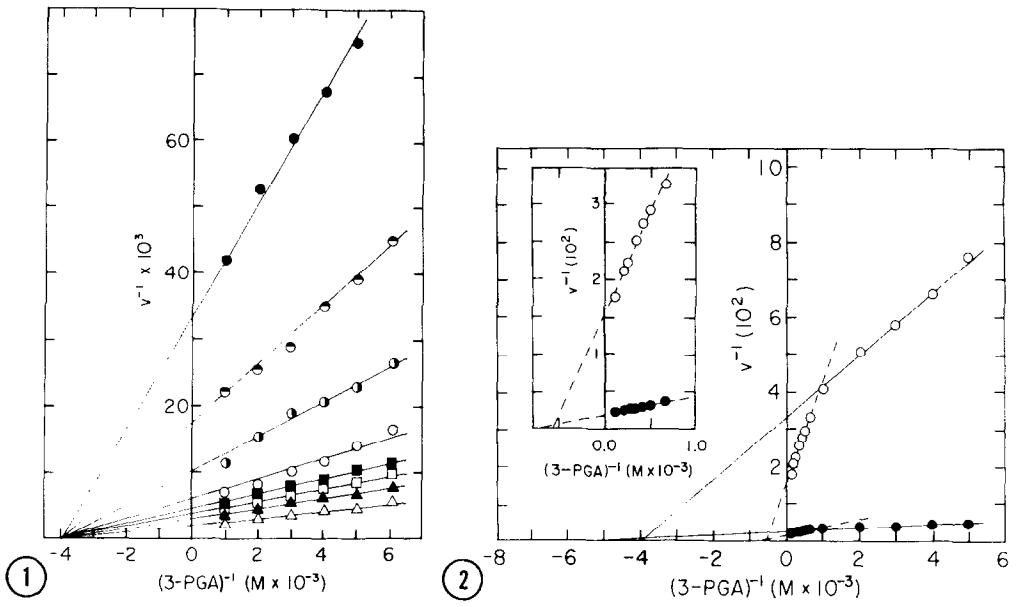

Fig. 1: The inhibition of phosphoglycerate mutase by commercial 3-PGA in the absence of added 2,3-DPG. Initial reaction velocities were obtained as described under Materials and Methods. Incubation mixtures contained the following percentages of commercial substrate: 100, ●; 99.7, ◐; 99, ◑; 97, ○; 95, ■; 90, □; 80, ▲; and 0, △. Different levels of 3-PGA purity were obtained by mixing commercial (contaminated) substrate with substrate purified by the process outlined under Materials and Methods. Most reaction mixtures contained 0.06 µg of purified mutase. In the presence of 100% commercial substrate, 0.22 µg of enzyme was used so that appropriate velocity measurements could be made.

Fig. 2: The activation of phosphoglycerate mutase by 3-PGA in the absence of added 2,3-DPG. Measurements were made in the presence of commercial (○) and purified (●) 3-PGA as described under Materials and Methods. The inset represents an enlarged replot of the data obtained at high substrate concentrations.

Normally, a contaminating inhibitor associated with a substrate will not manifest itself through a family of curves resembling classical noncompetitive inhibition as shown in Fig. 1 (24). Thus the nature of the observed inhibition is enigmatic and, therefore, must await further analysis.

As depicted in Fig. 2, relatively high levels of 3-PGA in the absence of 2,3-DPG produce biphasic double reciprocal plots. Thus at concentrations in excess of 1 mM, both commercial and purified 3-PGA accelerate mutase catalysis. This observation suggests that D-glycerate-3-P is capable of occupying two sites on the enzyme's surface, the normal catalytic site and an allosteric activator site.

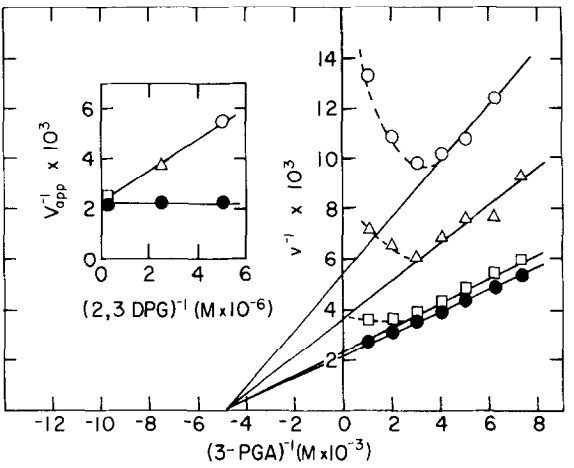

Fig. 3: Phosphoglycerate mutase initial reaction velocities obtained with commercial (open symbols) and pure (solid circles) 3-PGA in the presence and absence of 2,3-DPG (O, 0.2; △, 0.4; □, 4.0 μM). Solid circles (●) represent the average results obtained in the presence and absence of the above levels of 2,3-DPG. Measurements were performed as indicated in the previous figures.

Since the data in the preceding figures unequivocally demonstrate that erythrocyte phosphoglycerate mutase activity occurs in the absence of added 2,3-DPG, we were curious to know whether the addition of cofactor would influence the enzyme's catalytic capacity. Thus we initiated a kinetic study which in essence duplicated an investigation of red cell mutase recently reported by Ikura et al. (25). The latter group, however, used commercial 3-PGA throughout the entire course of its investigation[1]; we did not.

In Fig. 3, the reciprocal plots generated by the open symbols represent kinetic analyses conducted at three different fixed levels of 2,3-DPG (0.2-4.0 μM) in the presence of commercial 3-PGA. On the other hand, the line generated by the solid circles is a composite of four superimposable plots obtained by varying purified 3-PGA both in the presence and the absence of the aforementioned levels of 2,3-DPG. Thus, when purified D-glycerate-3-P is used as the variable substrate, 2,3-DPG has no influence on the kinetics of the

[1]In their study, Ikura et al. (17) used D-glycerate-3-P purchased from Boehringer-Mannheim. When this material is employed without further purification, it strongly inhibits the phosphoglycerate mutase reaction. L.F. Hass and K.B. Miller, unpublished data.

Table 1: Phosphoglycerate Mutase Kinetic Constants

Substrate	V_{max}	K_m	K_a
	U/mg	M	M
3-PGA (impure)	0.31×10^{2}	2.8×10^{-4}	1.9×10^{-3}
3-PGA (impure) + 2,3-DPG	4.5×10^{2} ‡	2.0×10^{-4}	---
3-PGA (pure) + 2,3-DPG	4.5×10^{2}	2.0×10^{-4}	1.3×10^{-3}

‡ Extrapolated value.

mutase reaction. In contrast, the inhibitor contained in commercial 3-PGA creates a dramatically different picture in the presence of 2,3-DPG. As shown, various levels of the latter substance generate a family of reciprocal curves which Ikura et al. (25) suggest as being indicative of a sequential reaction mechanism involving the formation of a ternary complex. In light of the data presented here, however, 2,3-DPG performs as a nonessential activator which is capable of overcoming a mixed "noncompetitive/substrate" inhibition. Thus with increasing concentrations of 2,3-DPG, the curvilinearity at high 3-PGA levels disappears, and the slopes and intercepts of the inhibited reciprocal plots approach limiting values which are equivalent to those of the plot generated through the use of purified 3-PGA alone.

The various kinetic constants derived from preceding figures are presented in Table 1. Regardless of its state of purity, 3-PGA manifests a K_m value (0.20-0.28 mM) which is in excellent accord with those previously reported for the enzymes obtained from muscle (26,27), red cell (25), wheat germ (28), and yeast (4).

In an effort to determine the nature of the inhibitor associated with commercial 3-PGA, we have ruled out D,L-glycerate (1-300 μM), P_i (1-10 μM), Ba^{2+} (10 mM), and glycolate-2-P (200 μM). None of these substances cause any appreciable inhibition when combined with purified 3-PGA as the sole substrate in the reaction mixture.

DISCUSSION

For the first time, evidence is presented which indicates that a phosphoglycerate mutase from higher animals does not require added 2,3-DPG in order to function normally. Although the mechanism of the reaction described in this paper remains obscure, reports relating to the formation of phosphoryl mutases from plant (7,12) and animal (8,10,11) sources make it likely that the human red cell enzyme is transformed into a kinetically competent phosphoryl intermediate during catalysis.

Since we have shown that erythrocyte phosphoglycerate is readily phosphorylated by 2,3-DPG (15)[2], it appears that both the latter compound and 3-PGA may be equally capable of forming E-P, suggesting that phosphoryl transfer to acceptor rather than enzyme phosphorylation is the rate-limiting step in the mutase reaction. Thus each of the schemes described in the Introduction may have some foundation in reality.

In many cases, a contaminating inhibitor associated with substrate may go undetected and, therefore, give kinetic results which are totally deceptive. This appears to have been the case with Ikura et al. (25), leading them to propose that red cell mutase manifests a sequential mechanism. In view of our findings, however, a completely different perspective emerges, bringing the enzyme's catalytic properties more into accord with those generally ascribed to other phosphomutases.

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[2] When human erythrocyte phosphoglycerate mutase is pulse-labeled with 5 mM [U-^{32}P]2,3-DPG in 10 mM HEPES-2 mM mercaptoethanol (pH 7.4), the enzyme acquires 2 moles of covalently bound ^{32}P per mole of protein. L.F. Hass and K.B. Miller, unpublished data.

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