A REASSESSMENT OF THE PHOSPHOGLYCERATE BYPASS ENZYMES IN HUMAN ERYTHROCYTES

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SUMMARY: Dissociation of the human erythrocyte into cytoplasmic and membranous components, shows that all of the cell's intrinsic 2,3-diphosphoglycerate phosphatase activity is associated with the soluble component. Further fractionation of the cytoplasm on DEAE cellulose illustrates that both 1,3-diphosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase activities occur coincidently within one peak. Thermal denaturation of the peak proteins at 60° results in a parallel loss in phosphatase and mutase activity. The identical phenomenon is observed in the presence of the 2,3-diphosphoglycerate phosphatase activator, 2-phosphoglycolate. Homogeneous 1,3-diphosphoglycerate mutase, which quantitatively accounts for all of the intrinsic 2,3-diphosphoglycerate phosphatase within the red cell, also exhibits thermal instability at 60°. These findings suggest that the phosphoglycerate bypass in erythrocytes is under the control of a single, bifunctional enzyme.

2,3-Diphosphoglycerate (2,3-DPG) is the most abundant organophosphate found in human erythrocytes (1). The compound apparently plays a key role in both the control of red cell metabolism (2) and in the regulation of hemoglobin oxygen association (3,4). On the basis of early studies, Rapoport and Luebering (5,6) proposed that intracellular levels of 2,3-DPG are maintained by two specific enzymes: 1,3-diphosphoglycerate mutase (EC 2.7.5.4) and 2,3-diphosphoglycerate phosphatase (EC 1.3.13). Of the two enzymes, only the former has recently been purified to homogeneity from human erythrocytes (7,8). It is noteworthy, however, that partially purified red cell diphosphoglycerate phosphatase has been found to have certain catalytic characteristics identical to those ascribed to either or both of the phosphoglycerate mutases (i.e., mono- or diphosphoglycerate mutase) (9-12).

Highly purified preparations of diphosphoglycerate mutase and monophosphoglycerate mutase (EC 2.7.5.3) from diverse sources have been reported to

intrinsically manifest 2,3-diphosphoglycerate phosphatase activity (7,13-16). It has been previously shown that in the presence of the substrate analog, glycolate-2-P, this activity becomes magnified to the point that each of the above enzymes is essentially converted to a phosphatase and, for the most part, ceases to function as a mutase (7,16,17).

This paper deals with an exhaustive search in human erythrocytes for a specific 2,3-diphosphoglycerate phosphatase, devoid of other enzymatic activity. Our results show that if such an enzyme exists as part of either the membranous or the cytoplasmic milieu, its catalytic capacity is sufficiently low so that it cannot be detected. On the other hand, the most readily discernable phosphatase activity always occurs in conjunction with phosphoglycerate mutase activity. In fact, the most potent 2,3-diphosphoglycerate phosphatase activity is an intrinsic property of the enzyme, 1,3-diphosphoglycerate mutase. This suggests that the phosphoglycerate bypass in red cells is under the control of a single, bifunctional catalyst.

MATERIALS AND METHODS

Fresh human blood was obtained from The Hershey Medical Center blood bank. The blood was stored at 4° and was processed within 48 hr after collection.

Protein Determination: Protein was determined by the method of Lowry *et al.* (18), using bovine serum albumin as a standard (19).

Enzyme Assays: Diphosphoglycerate mutase was assayed at 25° by the method of Rapoport and Leubering (20) as modified by Rose (21). Monophosphoglycerate mutase activity was measured at 25° by the enolase-coupled assay of Grisolia (22) and Harkness *et al.* (23).

2,3-Diphosphoglycerate phosphatase activity was determined by measuring the release of ^{32}Pi from [U-^{32}P]2,3-DPG at 37°. The standard assay mixture was comprised of: 50 mM imidazole-acetate pH 6.5, 2 mM 2-mercaptoethanol, 2 mM K_2PO_4, 0.2 mM [U-^{32}P]2,3-DPG, and sufficient enzyme so that no more than 20% of the substrate was consumed during the reaction time. ^{32}Pi was counted in plastic vials containing 10 ml of scintillation fluid (24). Phosphatase activities were also performed in the presence of 0.1 mM glycolate-2-P. The specific activity of the [U-^{32}P]2,3-DPG was determined at the time of each experiment.

One unit of enzyme activity is defined as that amount of protein which effects the conversion of one µmole of substrate to product in one min.

Preparation of [U-^{32}P]2,3-DPG: Radioactive [U-^{32}P]2,3-DPG was obtained by adding 5 mCi of carrier-free ^{32}Pi to an incubation mixture containing: 4.5 mM DL-glyceraldehyde-3-P, 0.5 mM NAD, 1.3 mM D-glycerate-3-P, 5.1 mM pyruvate, 3.9 mM K_2HPO_4, 50 mM glygly, and 1 mM dithiothreitol. The mixture was brought to pH 7.5 and the volume adjusted to 40 ml. The reaction was started by adding

the following enzymes in 10 ml of glygly (pH 7.5): 2 mg of glyceraldehyde 3-phosphate dehydrogenase, 0.25 mg of lactate dehydrogenase, and ∿50 units of 1,3-diphosphoglycerate mutase. After 20 min, the reaction was stopped by the addition of 12 ml of 2 N $HClO_4$ and the resultant precipitate was removed by centrifugation. The supernatant solution was heated for 15 min at 100° to hydrolyze acid-labile phosphoryl compounds. The solution was cooled to 0°, and was neutralized with KOH. The $KClO_4$ precipitate was removed by centrifugation, carrier 2,3-DPG was added to the supernatant solution, and the solution was applied to an AG-1-Cl-X8 column (2.6 x 8 cm) equilibrated with 0.02 N HCl. After washing, the column was eluted with a 600 ml linear gradient ranging from 0.02 N to 0.12 N HCl. Fractions containing [U-^{32}P]-2,3-DPG were concentrated, were neutralized with NaOH, and were quantitated by the method of Rose and Liebowitz (17). Approximately 170 µmoles of 2,3-DPG were recovered with a specific activity of 1.18 x 10^7 cpm per µmole.

Preparation of Various Erythrocyte Fractions: All of the following operations were performed at 4°.

Hemolysate: Erythrocytes were separated from plasma by centrifuging at 7,000 x g for 20 min. The resultant cells were washed 3 times with 154 mM NaCl and were subsequently hemolyzed by adding an equal volume of distilled H_2O. The hemolysate was finally diluted 1:1 with 10 mM K-PO_4 - 4 mM mercaptoethanol (pH 7.0).

Membranes: Cell membranes were prepared in the presence of 5 mM K-PO_4 - 2 mM mercaptoethanol (pH 7.0) by the method of McDaniel *et al.* (25).

Cytoplasm: Cytoplasm was obtained by centrifuging membranes from the erythrocyte hemolysate (see above) at 60,000 x g for 1 hr.

RESULTS

During a search for a specific 2,3-diphosphoglycerate phosphatase in human erythrocytes, our approach was essentially three-pronged: (a) we initially separated the red cell into membranous and cytoplasmic components, and subsequently assayed each component for phosphatase activity both in the presence and the absence of the activator, glycolate-2-P, (b) the cellular component capable of catalyzing the hydrolysis of 2,3-DPG was further fractionated and all fractions were surveyed for phosphatase, monophosphoglycerate and diphosphoglycerate mutase activities, and (c) the thermal denaturation characteristics of the phosphatase-rich fractions were then compared with those associated with the red cell phosphoglycerate mutases which had been previously purifed to homogeneity in our laboratory (7).

When the human erythrocyte is hemolyzed and separated into cytoplasmic and membranous fractions, all of the 2,3-diphosphoglycerate phosphatase activity has been found to be associated with the cytoplasm. In the presence of 0.1 mM

glycolate-2-P, the cytoplasmic phosphatase activity increases about 400-fold whereas the membrane component is found to remain completely inert with regard to 2,3-DPG hydrolysis.

When erythrocyte cytoplasm is subjected to DEAE cellulose column chromatography as illustrated in Fig. 1, several well-defined protein peaks are obtained. The two early peaks are those which are not retarded by the adsorbant and the largest of these is primarily hemoglobin. As shown in Fig. 1A, the application of a linear KCl gradient to the column produces a well delineated separation of monophosphoglycerate from diphosphoglycerate mutase. Upon surveying the chromatographic fractions for 2,3-diphosphoglycerate phosphatase in the absence (Fig. 1B) and the presence (Fig. 1C) of glycolate-2-P, it is seen that the only detectable activity in both cases occurs exclusively in those fractions which contain diphosphoglycerate mutase activity.

It is known from additional studies conducted in our laboratory (Sheibley and Hass, unpublished data) that erythrocyte monophosphoglycerate mutase possesses intrinsic 2,3-diphosphoglycerate phosphatase activity which is greatly enhanced by glycolate-2-P. This activity is not apparent upon examination of Fig. 1. Thus, under the experimental conditions cited, it is obvious that we would be incapable of detecting heretofore unknown phosphatases, comparable in catalytic strength to that manifested by monophosphoglycerate mutase. Barring unanticipated biological phenomena, however, it seems reasonable to assume that the phosphatase activity shown in Fig. 1 represents the most potent red cell capacity for the hydrolysis of 2,3-DPG.

An investigation of the catalytic properties of homogeneous erythrocyte diphosphoglycerate mutase by Kappel *et al.* (7) revealed that the enzyme has the intrinsic capacity to hydrolyze 2,3-DPG both in the absence and the presence of glycolate-2-P. The phosphtase-stimulated activity of diphosphoglycerate mutase by glycolate-2-P had been previously demonstrated by Rose and Whalen (16), Rosa *et al.* (12), and most recently, by Sasaki *et al.* (8). Thus, we decided to measure the rate of thermal inactivation of the phosphatase and the diphospho-

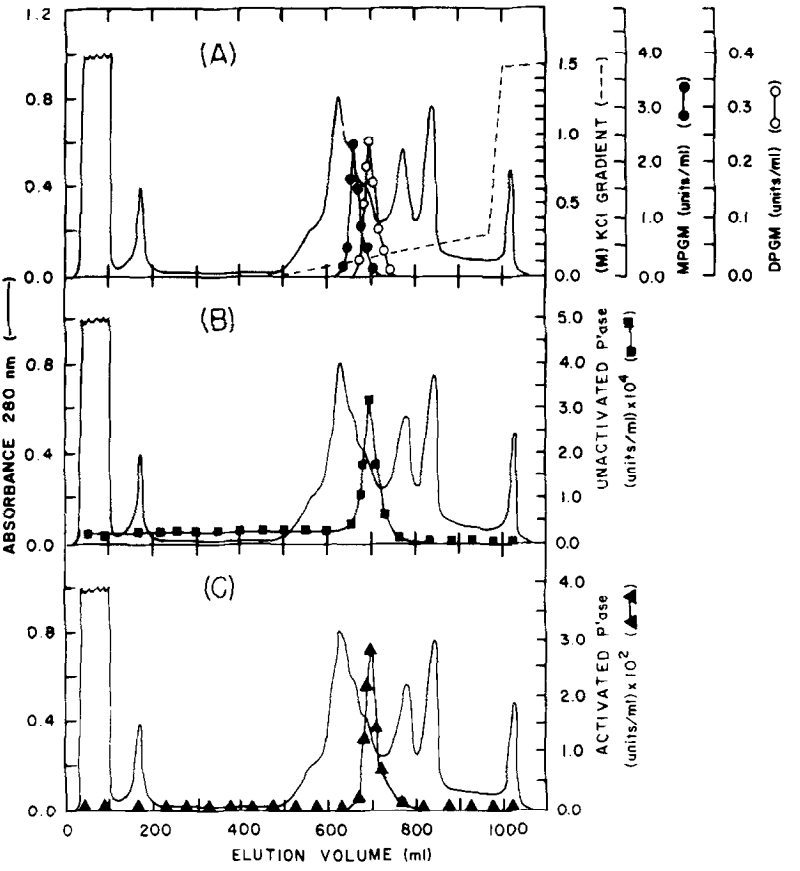

Fig. 1. Chromatography of erythrocyte cytoplasm on DEAE cellulose. The cytoplasm from 10 ml of packed red cells was applied to a 1.5 x 30 cm Whatman DE-52 column. The column was equilibrated with 5 mM $K\text{-}PO_4$ - 2 mM mercaptoethanol (pH 7.0) and had a flow rate of 50 ml per hr. Initially, the column was washed with 500 ml of buffer and was then eluted with a 500 ml buffered KCl gradient, ranging from 0.0-0.3 M (----). This was followed by a 250 ml stepwise elution with 1.5 M KCl - 5 mM $K\text{-}PO_4$ - 2 mM mercaptoethanol. Fractions (4.0 ml) were analyzed for absorbance at 280 nm (——).

(A) illustrates those fractions containing monophosphoglycerate (●) and diphosphoglycerate mutase (○) activity. (B) represents fractions manifesting intrinsic 2,3-diphosphoglycerate phosphatase activity (■), while (C) represents activated 2,3-diphosphoglycerate phosphatase activity obtained in the presence of 0.1 mM glycolate-2-P (▲).

All chromatographic operations were performed at 4°. Enzymatic activities were measured as described under Materials and Methods.

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glycerate mutase isolated by DEAE cellulose chromatography (Fig. 1). Activity loss was compared with that obtained with purified diphosphoglycerate mutase, exposed to identical denaturing conditions. As illustrated in Fig. 2, the rate

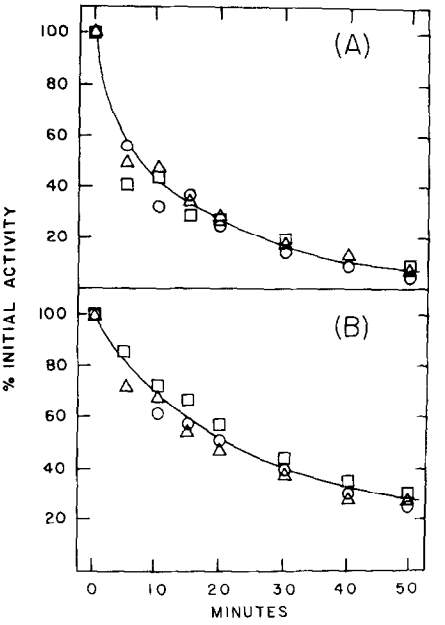

Fig. 2. Concurrent heat inactivation of diphosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase. Samples were heated at 60° in 140 mM KCl - 5 mM K-PO_4 - 2 mM mercaptoethanol, pH 7.0. Aliquots were removed at designated times and were immediately immersed in ice. Each aliquot was subsequently assayed for diphosphoglycerate mutase activity (○). 2,3-Diphosphoglycerate phosphatase activity was determined in the absence (□) and the presence (△) of 0.1 mM glycolate-2-P.

(A) represents the thermal inactivation of the fraction in Fig. 1 containing the peak phosphatase activity. (B) represents the inactivation of homogeneous diphosphoglycerate mutase, purified by Kappel *et al.* (7). Enzymatic activities were determined as described under Materials and Methods.

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of phosphatase inactivation at 60° was accompanied in each case by a parallel loss in dimutase activity. The fact that the same phenomenon was observed with both crude (Fig. 2A) and homogeneous (Fig. 2B) enzyme preparations, strongly indicates that the human red cell contains a single protein capable of regulating both the synthesis and the degradation of 2,3-DPG.

DISCUSSION

Through the pioneering efforts of Rose *et al.* (9,16,21,26), and to a lesser extent through the work of others (10,11,13), much has been learned about the enzymes responsible for the metabolism of 2,3-DPG within the human red cell. Of prime importance is the observation that certain anions (e.g., Cl^-, Br^-,

HPO_4^{2-}, SO_3^{2-}, etc.) behave as relatively moderate activators of the hydrolysis of 2,3-DPG. The most potent activator, however, is glycolate-2-P which greatly stimulates the erythrocyte's intrinsically low phosphatase capacity (1 nmole Pi released/min/ml packed cells at 37°) by several orders of magnitude (9). Interestingly, the presence of glycolate-2-P as a normal constituent of red cells remains to be conclusively established (27,28).

Rose and Whalen (16) have shown that glycolate-2-P induces purified diphosphoglycerate mutase to emulate a specific phosphatase which is capable of promoting the release of one mole of Pi from 2,3-DPG. From this observation it can be concluded that in the presence of glycolate-2-P, the phosphatase activity proposed by Rapoport and Leubering is augmented by the induced hydrolytic activity of the dimutase. Recently, Rosa *et al.* (12) and Sasaki *et al.* (8) have corroborated the glycolate-2-P effect on diphosphoglycerate mutase and, as a result, both groups have proposed that *in vivo* 2,3-DPG regulation may be directed by one enzyme. This hypothesis, however, is based on the premise that the erythrocyte contains only one 2,3-diphosphoglycerate phosphatase which is activated by a metabolite of questionable existence, as pointed out above.

This report conclusively shows that all of the intrinsic 2,3-diphosphoglycerate phosphatase activity of the human erythrocyte is associated exclusively with the cell's soluble component. When the data are analyzed for total units of activity per ml of packed cells, we obtain values ranging from 7×10^{-4} to 9×10^{-4} which match almost precisely the value reported previously by Rose and Liebowitz (9).

Analysis of the intrinsic (unactivated) phosphatase activities of homogeneous preparations of di- and monophosphoglycerate mutase, respectively, yields values of 9×10^{-4} and 1.5×10^{-4} units per ml of packed cells at pH 7.5 and 37° (Kappel and Hass; Sheibley and Hass, unpublished data). Hence, the above values show that practically all of the erythrocyte's diphosphoglycerate phosphatase activity is associated with the dimutase molecule. This concept is particularly reinforced by the findings illustrated in Figs. 1 and 2. Diphos-

phoglycerate mutase, therefore, apparently is the prime enzyme involved in the regulation of 2,3-DPG metabolism within the red cell. The exact mechanism employed by the integrated organism to control 2,3-diphosphoglycerate levels under various physiological and pathological conditions, however, remains an enigma; nevertheless, the concept of one enzyme acting as a "switch" for the control of intracellular concentrations of a given metabolite has definite heuristic appeal.

Despite its questionable role in 2,3-DPG metabolism, glycolate-2-P exerts a profound influence on the phosphatase activity of both red cell phosphoglycerate mutases. According to Table 1, 0.1 mM glycolate-2-P at pH 6.5 increases the specific phosphatase activities of the dimutase and the monomutase 340- and 9-fold, respectively. Thus, glycolate-2-P has an unusually

Table 1. The Influence of Glycolate-2-P on the 2,3-Diphosphoglycerate Phosphatase Activity of Diphosphoglycerate and Monophosphoglycerate Mutase.

Additions	Diphosphoglycerate Mutase		Monophosphoglycerate Mutase	
	Specific activity (U/mg)	Total activity (U/ml packed cells)	Specific activity (U/mg)	Total activity (U/ml packed cells)
None	0.008	1.1×10^{-3}	0.011	1.5×10^{-4}
0.1 mM Glycolate-2-P	2.69	3.8×10^{-1}	0.100	1.4×10^{-3}

The total units per ml of packed cells for monophosphoglycerate and diphosphoglycerate mutase activities were obtained from the data presented in Fig. 1A. Knowing the specific mutase activities of the purified enzymes obtained under identical conditions (diphosphoglycerate mutase = 5.61 and monophosphoglycerate mutase = 416 U/mg) (Kappel and Hass; Sheibley and Hass, manuscripts in preparation), the total mg of each protein per ml of packed cells was calculated. The specific 2,3-diphosphoglycerate phosphatase activities of homogeneous di- and monomutase preparations in the presence of 80 μM 2,3-DPG at pH 6.5 and 37° were determined with and without the addition of glycolate-2-P by the procedure described in Methods. The total phosphatase activity of each enzyme was then ascertained, using the appropriate mg per ml of packed cells.

large effect on the catalytic properties of diphosphoglycerate mutase and a relatively slight effect on the properties of monophosphoglycerate mutase. The relevance of this finding is uncertain, but it could indicate that the dimutase is more sensitive to the influence of *in vivo* regulators than the monomutase. Using the total activities of both mutases to calculate enzyme levels within the erythrocyte, it is found that diphosphoglycerate mutase (∿133 μg/ml packed cells) exceeds that of monophosphoglycerate mutase (∿13.9 μg/ml packed cells) by approximately an order of magnitude. The high 2,3-diphosphoglycerate phosphatase activity of the dimutase in the absence of glycolate-2-P, therefore, is ascribable solely to a concentration effect; however, in the presence of glycolate-2-P, the high phosphatase activity is due to an unusual activator effect augmented by a concentration effect.

Whether a natural activator, comparable to glycolate-2-P, occurs within the red cell remains to be determined. Nevertheless, an elucidation of the mechanism whereby glycolate-2-P exercises its influence on phosphoglycerate mutases could give some insight into the metabolic control of 2,3-DPG.

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