PURIFICATION AND PROPERTIES OF 2, 3-DIPHOSPHOGLYCERIC ACID PHOSPHATASE FROM HUMAN ERYTHROCYTES *

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Received February 21, 1969

SUMMARY

A 1200-fold purification of an enzyme from human erythrocytes which hydrolyzes 2,3-diphosphoglyceric acid (2, 3-DPG) to Pi and 3-phosphoglyceric acid (3-PGA) is described. The preparation is free of acid phosphatase and phosphoglycerate mutase. Enzyme activity is enhanced 37-fold by 20mM bisulfite. Dithionite and cyanide also cause stimulation. The pH optimum is 7.5 and the K_m for 2,3-DPG is $4x10^{-5}$M. Enzyme activity is inhibited by both 3-PGA and 2-PGA. It is believed that this enzyme, and not PGA mutase which in the presence of inorganic pyrophosphate has this same phosphatase activity, is the physiological intracellular phosphatase for 2,3-DPG in erythrocytes.

INTRODUCTION

Most mammalian erythrocytes including those of man contain large quantities of 2,3-diphosphoglyceric acid (2,3-DPG), several orders of magnitude greater than the amount required as cofactor for the enzyme phosphoglycerate mutase. Although the function of this large amount of 2,3-DPG has not been definitely established, several studies suggest that it maintains regulatory control over the activity of certain enzymes including hexokinase (1), adenylate deaminase (2), transaldolase and transketolase (3), and over the oxygenation of hemoglobin (4,5).

The mechanisms whereby the erythrocyte maintains the high concentration of 2,3-DPG are poorly understood. The pathways for its synthesis from 1,3-diphosphoglyceric acid and its degradation to phosphoglycerate (PGA) and P_i were described by Rapaport and Luebering (6,7). A method of purification and some properties of diphosphoglycerate mutase from erythrocytes have

* Work supported by Veterans Administration and National Institutes of Health grant # AM-09001-05.

recently been reported by Rose (8). Little has been reported on the enzymology of the catabolism of 2,3-DPG. Mányai and Várady (9) described the enhancement of 2,3-DPG degradation to pyruvate and P_i in intact red cells and hemolysates by bisulfite. We have purified and separated two enzymes from human erythrocytes which are specific phosphatases for this compound. The activity of one enzyme is greatly enhanced by inorganic pyrophosphate (PP_i). Details of studies on this enzyme, shown to be the same as phosphoglycerate mutase, are being reported elsewhere (10). The second enzyme, undoubtedly that described by Mányai and Várady (9), is stimulated by bisulfite. Methods for separation of the two enzymes, and some properties of this second enzyme are reported here.

MATERIALS AND METHODS

Preparation of P^{32}-labelled 2,3-DPG and details of the phosphatase assay have already been described (11). The PP_i, used previously for enhancing enzymatic activity, was replaced with 20 mM sodium bisulfite in these studies. Phosphoglyceric acid was measured enzymically (12) and inorganic and ash phosphates after Ames and Dubin (13). Phosphoglycerate mutase was measured spectrophotometrically at 240 mμ (14) and protein by the method of Lowry, et al. (15).

Human erythrocytes were washed three times with four volumes of cold 0.15 M NaCl and the buffy coat removed by aspiration. Cells, 100 ml, were hemolyzed in a 300 ml mixture of an equal volume of each of the following: 0.001 M EDTA, 0.005 M Tris chloride buffer, pH 7.4; and 0.001 M mercaptoethanol. DEAE cellulose, 5 g, was added and the mixture stirred for three hours at 5°. The cellulose with adsorbed enzyme was washed twice with the hemolyzing mixture and then eluted by stirring for 20 min. with 200 ml hemolyzing mixture containing 0.25 M KCl. The cellulose was filtered on a Büchner filter and rinsed with the same buffer. The protein in the filtrate was precipitated with 2 volumes of acetone (-20°). The precipitate was allowed to settle at -20°, the clear supernatant decanted and the precipitate collected by centrifugation. The precipitate was dissolved in 0.01 M KPO_4 buffer, pH 6.8,

containing 0.001 M EDTA and 0.001 M mercaptoethanol (starting buffer) and applied to a 1 x 20 cm column of hydroxylapatite (BioRad HTP). After washing with starting buffer the column was eluted with a linear gradient consisting of 250 ml starting buffer and 250 ml 0.15 M KPO_4^- buffer, pH 6.8, containing 0.001 M EDTA and 0.001 M mercaptoethanol. This step completely separated the PGA mutase from the bisulfite-stimulated 2,3-DPG phosphatase (Fig 1). Fractions containing the phosphatase were combined, dialyzed against 0.01 M Tris HCl buffer, pH 7.4, containing 0.001 M EDTA and 0.001 M mercaptoethanol. The enzyme was then applied to a 2 x 30 cm column of DEAE Sephadex G-50 and eluted with a linear gradient composed of 250 ml 0.01 M Tris HCl buffer, pH 7.4, containing 0.001 M EDTA and 0.001 M mercaptoethanol in the mixing chamber and 250 ml of 0.25 M KCl in the same buffer in the reservoir.

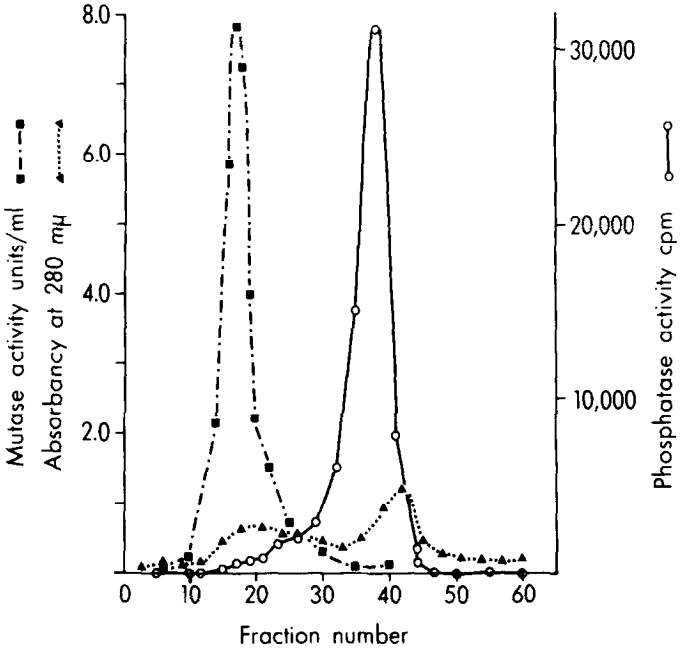

Fig 1. Chromatographic separation of PGA mutase and 2,3-DPG phosphatase on hydroxylapatite.

Details of application and elution are described in the text. Flow rate was 0.5 ml/min; fraction size, 5 ml. Assays containing 100 mM Tris chloride buffer, pH 7.6; 20 mM $NaHSO_3$; 0.33 mM 2,3-$D^{32}PG$ (5.6×10^6 cpm/μmole) and 0.01 ml enzyme in a volume of 0.5 ml were incubated for 60 min. at 37°.

TABLE I. Enzyme Purification

Step	Specific Activity μmoles/hr/mg protein	Purification Fold	Recovery %
1. Hemolysate	0.004	--	100
2. DEAE cellulose eluate	0.095	24	38
3. Hydroxylapatite	1.20	300	36
4. DEAE Sephadex	4.80	1200	22

TABLE II. Effect of various compounds on enzyme activity*

compound	activity (%)
sodium bisulfite	100
sodium dithionite	52
sodium borohydride	0
glutathione	11
cysteine	0
ascorbic acid	5
mercaptoethanol	3
dithiolerythritol (DTE)	5
bisulfite + DTE	106
ferrous sulfate	0
sodium cyanide	31
no addition	3

* All compounds 20 mM; activity is relative to activity with bisulfite.

This purification scheme resulted in a 1200-fold purification of the enzyme with 22 percent recovery. The enzyme contained negligible PGA mutase and no acid phosphate activity at pH 5.0 with p-nitrophenylphosphate as substrate.

RESULTS AND COMMENTS

The purified enzyme has optimal activity at pH 7.5. Bisulfite greatly enhances its activity. Optimal effect is achieved with 20-30 mM bisulfite, at which concentration the enzyme is stimulated 37-fold (Fig 2). Other compounds were tested for possible enhancement of enzyme activity (Table II). Of those tested, in addition to bisulfite only dithionite and NaCN caused significant stimulation. The stoichiometry of the reaction is given in Table III. For each mole of 2,3-DPG disappearing, one mole of P_i and one mole of phosphoglyceric acid appeared. The enzyme preparation used in this particular experiment contained PGA mutase and therefore it was not possible to assign the position of the phosphate ester. Subsequent studies using purified enzyme (step 4) showed that the product is 3-PGA.

TABLE III. Stoichiometry of 2,3-DPG phosphatase reaction

	μmoles
Decrease in 2,3-DPG	3.2
PGA formed	3.5
Pi formed	3.3

Reaction mixture, 10 ml, contained: 100 mM Tris chloride buffer, pH 7.5; 20mM $NaHSO_3$; 2.2mM 2,3-DPG (3.5×10^6 cpm/umole); 0.96 mg enzyme (600 fold purified).
To 5ml of reaction mixture at 0 time and after 3 hr at 37°, 1.0 ml 6% perchloric acid was added. 2,3-DPG was separated by chromatography on Dowex 1-Cl^- and quantitated by radioactivity. PGA and Pi were measured as described in text.

The apparent K_m of this enzyme for its substrate in the presence of bisulfite was found to be 4×10^{-5}M (Fig 3). It was the same without added bisulfite. This is a more favorable K_m than that of the PP_i-stimulated phosphatase which has a K_m for 2,3-DPG of 1.1×10^{-4}M (10).

Both 3-PGA and 2-PGA are potent inhibitors of the enzyme. When 0.33 mM 2,3-DPG was used in the assay approximately 50% inhibition was caused by 0.1 mM

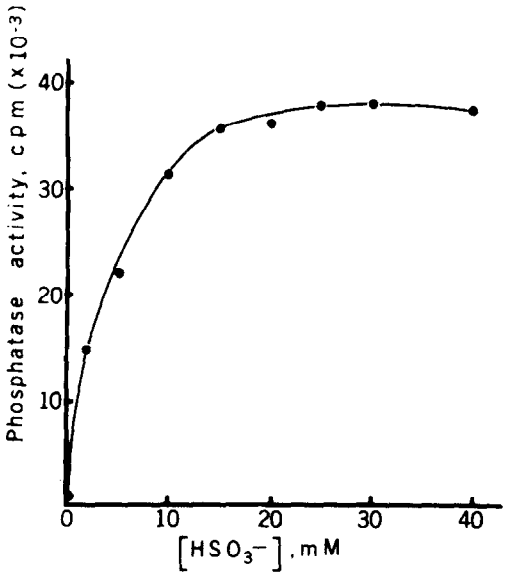

Fig 2. Effect of bisulfite concentration upon enzyme activity.

Incubation mixtures contained in a volume of 0.5 ml: 100 mM Tris chloride buffer, pH 7.8; 0.33 mM 2,3-D^{32}PG (5.3×10^6 cpm/umole); 4 μg enzyme (step 4); and bisulfite as indicated. Tubes were incubated 60 min at 37°.

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of either PGA. Inorganic phosphate failed to inhibit at such low concentrations but under the same conditions approximately 50% inhibition was attained with 15 mM P_i. The enzyme therefore has the characteristics of a specific phosphatase rather than a non-specific phosphatase.

It is clear from these studies that there are two separable enzymes in human erythrocytes capable of hydrolyzing 2,3-DPG to PGA and P_i. Whether both actually fulfill this function within the cell remains to be proven. It is known that intact erythrocytes are rapidly depleted of 2,3-DPG when placed in a medium containing bisulfite (9). Also in contrast to human red cells, caprine erythrocytes contain scarely any 2,3-DPG, diphosphoglycerate mutase, or the bisulfite-stimulated phosphatase (16). However these same cells contain nearly as much PGA mutase and PP_i-stimulated phosphatase as those from man. Because of these observations we believe the bisulfite-

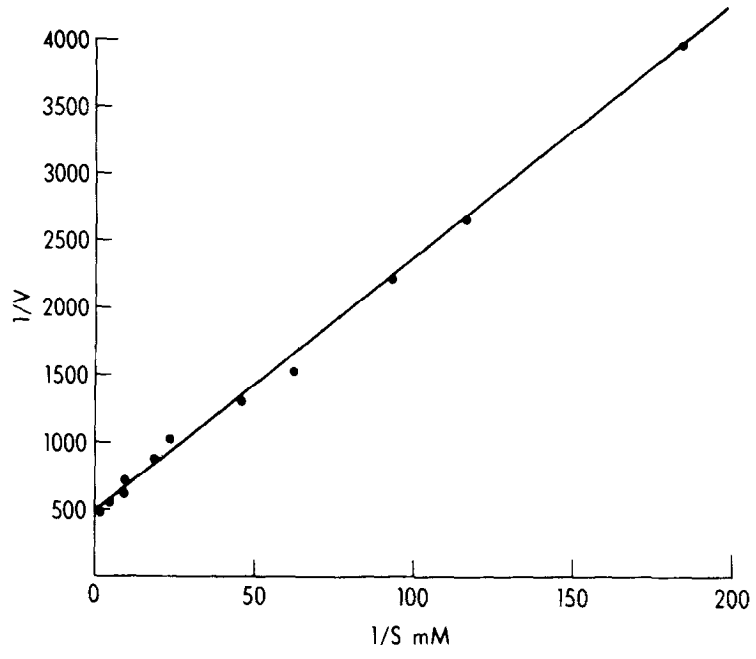

Fig 3. Effect of substrate concentration upon velocity of the reaction

Each 0.5 ml incubation mixture contained 100 mM Tris chloride buffer, pH 7.8; 20 mM sodium bisulfite; 4 μg purified enzyme (Step 4); and from 0.0054 to 0.54 mM 2,3-D^{32}PG (7.0 x 10^6 cpm/μmole). Incubation was for 60 min at 37°. Each point represents the average of three determinations. V, cpm x 10^{-7}.

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stimulated phosphatase is more likely to play the physiologic role as 2,3-DPG phosphatase within the cell. The levels of intracellular 3-PGA and 2-PGA may serve to partially regulate the activity of the enzyme. There may also be some intracellular mechanism for enzyme activation similar to that induced here by bisulfite. We are currently attempting to illucidate the mechanism of the remarkable degree of stimulation of this enzyme by bisulfite.

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