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The Isolation and Partial Characterization of Diphosphoglycerate Mutase from Human Erythrocytes†

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ABSTRACT: Diphosphoglycerate mutase has been purified to homogeneity from outdated human erythrocytes. The native enzyme has a molecular weight of 57 000 as determined by equilibrium centrifugation and exclusion chromatography. Disc gel electrophoresis in the presence of sodium dodecyl sulfate yields a single protein band with a molecular weight of about 26 500, indicating that diphosphoglycerate mutase is comprised of two subunits of similar mass. The enzyme exhibits the following intrinsic activities: diphosphoglycerate mutase, monophosphoglycerate mutase, and 2,3-diphosphoglycerate phosphatase. The latter activity is enhanced in the presence of either organic or inorganic anions. Glycolate-2-P, particularly, has a profound activating effect. Nonspecific phosphatase and enolase activities are absent. The enzyme has an extinction coefficient at 280 nm of 1.65 cm^2/mg. The amino acid composition of the homogeneous protein has been determined.

Rapoport and Luebering (1950, 1951) were the first investigators to propose that 2,3-diphosphoglycerate (2,3-DPG)[1] levels within the erythrocyte are maintained by two specific enzymes: bisphosphoglyceromutase (EC 2.7.5.4): diphosphoglycerate mutase, DPGM, and bisphosphoglycerate phosphatase (EC 3.1.3.13): diphosphoglycerate phosphatase, DPGP. After the physiologically important observation that the presence of 2,3-DPG profoundly influences the complexation between oxygen and hemoglobin (Chanutin and Curnish, 1967; Benesch et al., 1968), several attempts have been made to isolate each of the above red cell enzymes (Sasaki et al., 1975; Rose and Whalen, 1973; de Verdier and Groth, 1973; Harkness et al., 1970; Rose and Liebowitz, 1970). Recently, however, several reports have suggested that intracellular 2,3-DPG concentrations may be regulated by one protein, DPGM, which has been shown to manifest both mutase and phosphatase activity (Kappel et al., 1975; Hass and Miller, 1975; Sasaki et al., 1975; Rosa et al., 1973). If this is the case, the original Rapoport–Luebering proposal is incorrect; consequently, a separate DPGP is probably nonexistent.

In order to test the validity of the above hypothesis and to study the multifunctionality of DPGM, we have devised a relatively simple scheme for the isolation of homogeneous diphosphoglycerate mutase from outdated human erythrocytes.[2] Previously described methods for the purification of DPGM have resorted to the use of isoelectric focusing (Rose and Whalen, 1973; Sasaki et al., 1975), a technique, which in its current state of development, renders impractical the isolation of large quantities of enzyme. Our procedure, on the other hand, is limited solely by the availability of biological material.

In addition to describing a process for the purification of diphosphoglycerate mutase, this report contains data which both corroborate and supplement certain physicochemical characteristics of the DPGM molecule.

Experimental Procedures

Materials. Outdated human erythrocytes of various blood types were obtained from The Hershey Medical Center blood bank. NAD^+, carboxymethylcellulose, dithiothreitol, the diethyl acetal (barium salt) of DL-glyceraldehyde-

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[1] Abbreviations used are: 2,3-DPG, 2,3-diphosphoglycerate; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase; 3-PGA, D-glycerate-3-P; 2-PGA, D-glycerate-2-P; MPGM, monophosphoglycerate mutase.

[2] This report is an expansion of a preliminary communication by Kappel et al. (1975).

3-P, the sodium salts of D-glycerate-3-P (3-PGA), D-glycerate-2-P (2-PGA), D-glucose-6-P, β-glycerol phosphate, and the pentacyclohexylammonium salt of 2,3-diphospho-D-glycerate were purchased from Sigma. The tricyclohexylammonium salt of glycolate-2-P was purchased from General Biochemicals. Monophosphoglycerate mutase (MPGM) and enolase were obtained from Boehringer Mannheim. Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), bovine serum albumin, myoglobin (horse skeletal muscle), ovalbumin, cytochrome *c* (horse heart), and α-chymotrypsinogen A (bovine pancreas) were purchased from Sigma. DEAE-cellulose (DE-52, 1.0 mequiv/g) was obtained from Whatman. DEAE-cellulose (Cellex-D, 0.7 mequiv/g), hydroxylapatite (Bio-Gel HTP), and the ion-exchange resins AG-1-Cl-X8 (200–400 mesh) and AG50W-X2 (200–400 mesh) were purchased from Bio-Rad. Sephadex G-100 Superfine was obtained from Pharmacia. Enzyme grade ammonium sulfate was purchased from Schwarz/Mann. Carrier-free [^{32}P]phosphoric acid was obtained from New England Nuclear. All other chemicals were of reagent grade.

Enzyme Assays. All spectrophotometric assays were performed on a Cary Model 15 recording spectrophotometer. DPGM was assayed at 25° by a modified method of Rose (1968) using 1.0 ml of the following mixture: 25 mM glycylglycine (pH 7.8), 1.5 mM dithiothreitol, 1 mM K_2HPO_4, 0.24 mM DL-glyceraldehyde-3-P, 1 mM 3-PGA, and 0.5 mM NAD^+.

After purification, DPGM was assayed for enolase, nonspecific phosphatase, monophosphoglycerate mutase, and 2,3-DPG phosphatase activities. The following methods were employed.

Enolase. DPGM (50 μg) was assayed for enolase activity at 25° by the procedure of Holt and Wold (1961).

Nonspecific Phosphatase. DPGM (88 μg) was incubated at 37° in 2.5 ml of 50 mM glycylglycine–2 mM 2-mercaptoethanol (pH 7.8), containing 10 mM of either glucose-6-P or β-glycerol phosphate. At appropriate intervals, aliquots were assayed for liberated P_i (Fiske and Subbarow, 1925).

Monophosphoglycerate Mutase. MPGM activity was measured at 25° in 1.0 ml of the following incubation mixture: 35 mM Tris-HCl (pH 7.0), 20 mM 3-PGA, 2.5 mM 2,3-DPG, 2.5 mM $MgSO_4$, 20 μg of enolase, and 45 μg of DPGM. The conversion of 3-PGA to 2-PGA was related to the number of micromoles of phosphoenolpyruvate formed, using the extinction coefficient, $A_{240,PEP} = 1.11 \times 10^3\ M^{-1}\ cm^{-1}$ (Harkness et al., 1970).

2,3-DPG Phosphatase. The following solution (1.0 ml) was incubated at 37°: 50 mM imidazoleacetate (pH 6.5), 2 mM dithiothreitol, 0.08 mM [U-^{32}P]-2,3-DPG, and 10 μg of DPGM. At selected times, aliquots (0.2 ml) were added to 0.7 ml of 0.57 N H_2SO_4 and 0.1 ml of 5% ammonium molybdate. The solution was mixed and 0.25 μmol of carrier P_i was added. The released $^{32}P_i$ was extracted as the phosphomolybdate complex and was counted (Rose and Liebowitz, 1970).

One unit of enzyme activity is defined as that amount of protein which catalyzes the formation of 1.0 μmol of product/min under the conditions described.

Preparation of [U-^{32}P]-2,3-DPG. Radioactive [U^{32}P]-2,3-DPG was prepared from 5 mCi of carrier-free $^{32}P_i$ according to the method of Harkness et al. (1970). The reaction was stopped by the addition of 2 N $HClO_4$ and the resultant precipitate was removed by centrifugation. The isotopically labeled diphosphoglycerate was purified and was quantitated by the methods of Rose and Liebowitz (1970a,b). About 70 μmol of product was recovered. The percent labeling and the specific activity of the C-2 phosphate were determined to be 51% and 2.1×10^6 cpm/μmol, respectively (Rose and Pizer, 1968). D-Glyceraldehyde-3-P was quantitated by the procedure of Racker (1957).

Molecular Weight Determination. The native molecular weight of DPGM was estimated by exclusion chromatography on Sephadex G-100 Superfine as described by Andrews (1964). The column (2.5 × 95 cm) was equilibrated with 25 mM K-PO_4 (pH 7.0) and was then calibrated with the following globular proteins: cytochrome *c* (12 400), myoglobin (17 200), α-chymotrypsinogen A (25 000), ovalbumin (43 000), and bovine serum albumin (68 000).

The native molecular weight of DPGM was also determined by high-speed equilibrium centrifugation at 20°, according to the method of Yphantis (1964). Protein solutions were prepared by exhaustive dialysis against 50 mM K-PO_4 (pH 7.0)–2 mM 2-mercaptoethanol (ρ = 1.005 at 20°). Centrifugation was performed with 3.0-mm liquid columns, employing the multichanneled cell of Ansevin et al. (1970). Interference fringe patterns were measured with an automated microcomparator (Carlisle et al., 1974) and the effective reduced molecular weights [$\sigma_{n,w}(r)$] were estimated as a function of radial position within the cell (Roark and Yphantis, 1969). A partial specific volume of 0.733 ml/g (calculated from amino acid composition) was used in all determinations.

The subunit molecular weight of DPGM was determined using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis system of Weber et al. (1972). Gels were prepared from 10% acrylamide solutions.

Amino Acid Analysis. Amino acid analyses were performed in triplicate with a Beckman 120C autoanalyzer (Caban and Hass, 1971). Tryptophan content was determined by the method of Goodwin and Morton (1946) and half-cystine was determined by the procedure of Hirs (1956).

Extinction Coefficient. DPGM was exhaustively dialyzed against 5 mM K-PO_4 (pH 7.0) and the absorbance at 280 nm was determined. After Kjeldahl digestion, the micrograms of nitrogen per milliliter of enzyme solution was determined in quadruplicate by the Nessler procedure (Minari and Zilversmit, 1963). Assuming that the enzyme has a 16% nitrogen content, an extinction coefficient for a 0.1% DPGM solution at 280 nm was estimated to be 1.65 cm^2/mg.

Other Methods. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Hematocrits were determined with Wintrobe hematocrit tubes.

Enzyme Purification. All buffer solutions contained 2.0 mM 2-mercaptoethanol and were adjusted to pH at room temperature. Unless otherwise stated, all procedures were carried out at 0–5°.

Hemolysis. Red cells (7–9 units) were washed three times with 0.9% NaCl. The packed cells (98% hematocrit) were hemolyzed by the addition of an equal volume of water followed by occasional stirring for 15 min. One volume of 10 mM K-PO_4 (pH 7.0)–4 mM 2-mercaptoethanol was added to the hemolysate and the solution was filtered under vacuum, using a Büchner funnel and a filtering flask attached to a water aspirator.

Batch DEAE-Cellulose Elution. Cellex-D was equili-

Table I: The Purification of Human Erythrocyte Diphosphoglycerate Mutase.[a]

	Total Protein (mg)	Total Activity (units)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
1. Batch DEAE-cellulose elution	7475	876	0.12	100	
2. Ammonium sulfate fractionation (40–60%)	2930	674	0.23	77	1.9
3. First DEAE-cellulose chromatography	256	464	1.81	53	15.1
4. Second DEAE-cellulose chromatography	162	394	2.43	45	20.3
5. Hydroxylapatite chromatography	52	245	4.73	28	39.4
6. Heat treatment	49	237	4.81	27	40.1
7. Sephadex G-100 chromatography	34	193	5.61	22	47.8

[a] Enzyme activity was measured as outlined under Experimental Procedures. Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

brated with 5 mM K-PO_4 (pH 7.0) and was stored as a moist cake. The adsorbant was then added to the filtered hemolysate (1.5 g/10 ml) and the resultant suspension was stirred occasionally for 1 hr. The suspension was washed free of unadsorbed hemoglobin by vacuum filtration in the manner described above (Hennessey et al., 1962). Adsorbed protein was eluted from the ion-exchange cellulose by washing three times with 100 mM K-PO_4 (pH 7.0)–150 mM KCl. During each wash, 4 ml of buffer was used per g of DEAE-cellulose. The protein eluate was collected by vacuum filtration and was concentrated tenfold by ultrafiltration, using an Amicon apparatus equipped with a PM-10 membrane.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was slowly added to the concentrated protein solution to obtain 0.40 saturation at 0° (Dawson et al., 1969). The solution was maintained at approximately pH 7.0 (indicator paper) by the addition of dilute NH_4OH and was allowed to stand 1 hr prior to centrifugation at 9000*g* for 20 min. The supernatant solution was adjusted to 0.60 saturation with solid ammonium sulfate, allowed to stand for 1 hr, and was recentrifuged. The protein pellet was dissolved in 75 ml of 5 mM K-PO_4 (pH 7.0) and was exhaustively dialyzed against the same buffer.

First DEAE-Cellulose Chromatography. A CM-cellulose column (1.5 × 30 cm) was connected in series with a DEAE-cellulose (DE-52) column (2.5 × 35 cm) and both columns were equilibrated with 5 mM K-PO_4 (pH 7.0). The dialyzed ammonium sulfate fraction was centrifuged to remove any isoluble protein and was applied to the CM-cellulose column. The column complex was washed with 65 ml of the above buffer and was subsequently disassembled. The DEAE-cellulose column was further washed with one column volume of 25 mM K-PO_4 (pH 7.0) and was then eluted with a linear gradient, comprised of 700 ml of the wash buffer plus 700 ml of 125 mM K-PO_4 (pH 7.0). The elution rate was 40 ml/hr. All fractions (10 ml) containing DPGM activity greater than 1.0 unit/ml were pooled and the total protein was precipitated by the careful addition of solid ammonium sulfate (0.36 g/ml). The solution was centrifuged. The resultant protein pellet was dissolved in 25 ml of 25 mM K-PO_4 (pH 7.0), and was dialyzed against the solvent medium.

Second DEAE-Cellulose Chromatography. The dialysate was applied to a DEAE-cellulose (DE-52) column (1.5 × 15 cm) equilibrated with the above buffer. The protein was eluted with a linear gradient consisting of 300 ml each of the dialysis buffer and 125 mM K-PO_4 (pH 7.0). The flow rate was 30 ml/hr. Fractions (5 ml) containing DPGM activity greater than 2 units/ml were pooled. The total protein was precipitated as described above. The resultant precipitate was dissolved in 25 ml of 30 mM K-PO_4 (pH 6.8) and was dialyzed against the same buffer.

Hydroxylapatite Chromatography. The dialyzed solution was applied to a hydroxylapatite column (1.5 × 8 cm) which was subsequently washed with four column volumes of 30 mM K-PO_4 (pH 6.8). The elution rate was 20 ml/hr. DPGM was then eluted with 50 mM K-PO_4 (pH 6.8) and the fractions containing maximal activity were pooled.

Heat Treatment. The pooled DPGM fractions were then heated at 60° for 5 min. This was followed by cooling in ice and centrifugation at 12 000*g* for 30 min. The supernatant solution was decanted and its total protein was collected by precipitation with solid ammonium sulfate as described above.

Sephadex G-100 Chromatography. The protein from the previous step was dissolved in 2 ml of 25 mM K-PO_4 (pH 7.0) and was applied to a Sephadex G-100 column (2.5 × 90 cm). The enzyme was eluted at 7 ml/hr. Fractions (4 ml) were collected and were analyzed for both protein content and DPGM activity. Those fractions manifesting a constant DPGM specific activity were pooled and the total protein was precipitated by the addition of solid ammonium sulfate. The precipitate was dissolved in 25 mM K-PO_4 (pH 7.0)–4 mM 2-mercaptoethanol–5 mM EDTA to give a final concentration of about 5–7 mg of enzyme/ml. The solution was dialyzed against the above buffer and was subsequently stored in small aliquots at −60°. Under these conditions, the enzyme was stable for at least 2 months.

Results

Enzyme Purification. The isolation of DPGM from outdated human erythrocytes is summarized in Table I. Compared with previously published purification methods (Rose and Whalen, 1973; Sasaki et al., 1975), our process is decidedly less complex and yields a substantially greater amount of enzyme which, despite different assay conditions, appears to possess a relatively higher specific activity (see Discussion).

An added bonus of our procedure is that homogeneous monophosphoglycerate mutase can be obtained from the same batch of starting material (Kappel et al., 1975; R. H. Sheibley and L. F. Hass, manuscript in preparation). The critical step in separating DPGM from MPGM occurs during ammonium sulfate fractionation. Thus, most of the DPGM activity is collected at 0.4–0.6 $(NH_4)_2SO_4$ saturation (0°), whereas MPGM is salted out at 0.6–0.75 saturation.

In order to determine the homogeneity of DPGM purified by the above protocol, we resorted to three criteria: the

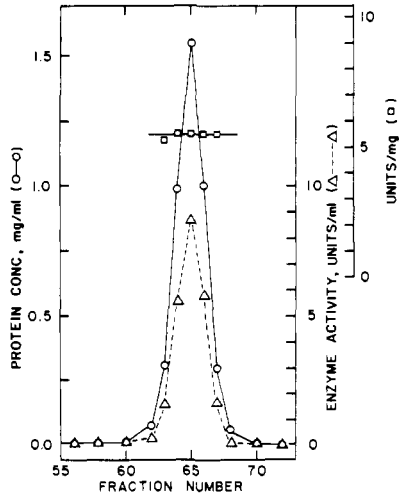

FIGURE 1: Chromatography of human erythrocyte diphosphoglycerate mutase on Sephadex G-100. Fractions (4 ml) were collected and assayed for both enzymatic activity and protein concentration, using the methods outlined under Experimental Procedures.

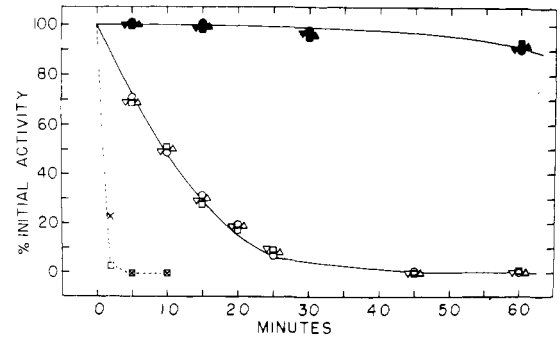

FIGURE 2: Rates of thermal inactivation of the intrinsic activities associated with purified erythrocyte diphosphoglycerate mutase. Open and solid symbols represent studies conducted at 65 and 60°, respectively. Activities measured include: diphosphoglycerate mutase (○), monophosphoglycerate mutase (□), 2,3-DPG phosphatase (▽), and activated 2,3-DPG phosphatase in the presence of 0.1 mM glycolate-2-P (△). The broken curve represents a control experiment conducted on purified monophosphoglycerate mutase alone at 60° (□) and in the presence of excessive amounts (250:1, w/w) of either bovine serum albumin or DPGM (X). Appropriate corrections were made for the intrinsic monophosphoglycerate mutase activity of the DPGM used in the control. Incubations were conducted in 25 mM K-PO_4–2 mM 2-mercaptoethanol (pH 7.0). DPGM and MPGM concentrations were 1.27 and 0.20 mg/ml in the initial and control experiments, respectively. Aliquots were removed at the indicated intervals and were quickly cooled in ice prior to assay by the methods given in Experimental Procedures.

generation, by exclusion chromatography, of one protein peak with constant specific activity; the presence of a single, sharp band upon polyacrylamide gel electrophoresis; and the complete lack of dependence of the macromolecular weight upon protein concentration after the establishment of sedimentation equilibrium. The first criterion is met in Figure 1 which illustrates that chromatography of DPGM on Sephadex G-100 yields a single protein peak of constant specific activity throughout its entire breadth. Although not shown, the same protein manifests a single, sharp band when 40 μg is subjected to polyacrylamide gel electrophoresis in either the presence or the absence of sodium dodecyl sulfate (criterion two). The last criterion is satisfied by Figure 3, which shows the reduced number and weight point-average molecular weights [$\sigma_{n(r)}$, $\sigma_{w(r)}$] (Yphantis, 1964) as a function of the protein concentration generated within the centrifuge cell during sedimentation equilibrium. The virtual invariance of the depicted molecular weights as well as the good agreement between the different point-average masses is strongly indicative of protein monodispersity.

Intrinsic Activities of DPGM. Employing the assay conditions cited under Experimental Procedures, purified diphosphoglycerate mutase has a specific activity of 5.61 U/mg at pH 7.8 and 25°. The purified enzyme also possesses as intrinsic activities both MPGM (1.18 U/mg at pH 7.0 and 25°) and DPGP (0.008 U/mg at pH 6.5 and 37°). DPGM apparently does not manifest either enolase or nonspecific phosphatase activity.

Evidence that MPGM and DPGP activities are intrinsic to the diphosphoglycerate mutase molecule is presented in Figure 2. As shown, the rate of thermal inactivation of DPGM at 65° in 25 mM K-PO_4–2 mM 2-mercaptoethanol (pH 7.0) exactly coincides with the rates of inactivation of both MPGM and DPGP.[3] At 60°, however, all of the above activities are remarkably stable, retaining 90% of their catalytic capacities after 60 min. On the other hand, homogeneous MPGM, isolated by the method of R. H. Sheibley and L. F. Hass (manuscript in preparation), loses all of its activity within 5 min at 60° under identical conditions. This phenomenon persists even in the presence of excessive amounts (250:1, w/w) of protective proteins such as bovine serum albumin or DPGM. The observation that purified MPGM suffers rapid inactivation in the presence of DPGM, which retains intrinsic MPGM capacity at 60°, strongly indicates the multifunctional nature of the DPGM molecule.

Rose and Liebowitz (1970) have shown that the 2,3-DPG phosphatase activity of human erythrocytes is stimulated by certain organic and inorganic anions. Recently, however, several reports indicate that 2,3-DPG levels within the red cell are regulated by one protein, DPGM, which (as illustrated in Figure 2) manifests both phosphatase and mutase activity (Kappel et al., 1975; Hass and Miller, 1975; Sasaki et al., 1975; Rosa et al., 1973). If DPGM, rather than a specific phosphatase, is indeed the responsible intracellular catalyst for the hydrolysis of 2,3-DPG, the purified enzyme should be capable of anionic activation under conditions similar to those cited by the aforementioned investigators. That this is the case is illustrated in Table II.

Physicochemical Characteristics. The molecular weight of native diphosphoglycerate mutase has been determined both by exclusion chromatography and by high-speed equilibrium centrifugation. Figure 3 shows the apparent weight- and number-average molecular weights (right-hand ordinate) obtained in low ionic strength neutral buffer at three different initial protein concentrations. In all cases, the mass of the DPGM molecule corresponds closely to 56 500. When the molecular weight is estimated by exclusion chromatography under slightly different solvent conditions (25 mM K-PO_4 (pH 7.0); see Experimental Procedures), a value of 57 000 is obtained.

Estimation of the molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gives a value of 26 500, indicating that DPGM is comprised of two subunits of similar mass.

The amino acid composition of human erythrocyte DPGM is presented in Table III. The final corrected value

[3] DPGP activity was determined both in the presence and the absence of glycolate-2-P, a potent stimulator of red cell 2,3-DPG phosphatase activity (Rose and Liebowitz, 1970).

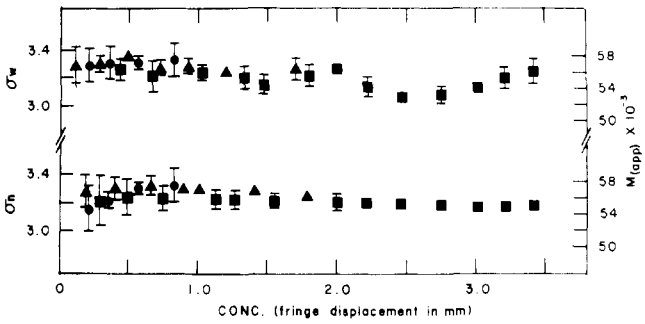

FIGURE 3: Determination of the native molecular weight of diphosphoglycerate mutase by high-speed equilibrium centrifugation. The left-hand ordinate depicts the effective reduced number- (σ_n) and weight-average (σ_w) molecular weight as a function of protein concentration within the centrifuge cell. The right-hand ordinate indicates the actual molecular weight. The experiment was conducted at 20° in 50 mM K-PO_4-2 mM 2-mercaptoethanol (pH 7.0), using a rotor speed of 22 000 rpm. Initial protein concentrations (C_0) were: 0.2 (●), 0.4 (▲), and 0.8 (■) mg/ml.

Table II: The Effects of Various Anions on the Intrinsic 2,3-Diphosphoglycerate Phosphatase Activity of Purified Diphosphoglycerate Mutase.[a]

Compound	Concn[b] (mM)	Velocity Relative to Standard Assay
None		1.0
PP_i	5.0	4.1
SO_3^{2-}	5.0	1.0
Cl^-	100.0	1.4
P_i	5.0	2.0
Cl^- plus P_i	100.0–5.0	7.2
Glycolate-2-P	0.1	430

[a] Assays were conducted in a total volume of 0.25 ml for 30 min at 37°. Incubation mixtures contained: 50 mM imidazoleacetate (pH 6.5), 2 mM 2-mercaptoethanol, 0.08 mM [U-^{32}P]-2,3-DPG (1.3 × 10^6 cpm/μmol), the indicated concentration of anion, and 5 μg of DPGM. In the presence of glycolate-2-P, DPGM was diluted 200-fold. Released $^{32}P_i$ was determined as described under Experimental Procedures. [b] Anion concentrations are those shown to stimulate the 2,3-DPG phosphatase activity of human erythrocytes (Rose and Liebowitz, 1970).

Table III: Amino Acid Composition of Human Erythrocyte Diphosphoglycerate Mutase.

Amino Acid Residue	Final Values[a] (μmol/mg)	Residues per 57 000 Molecule Weight
Lysine	0.369	27
Histidine	0.203	15
Arginine	0.479	33
Aspartic acid	0.708	48
Threonine	0.167	11
Serine	0.365	26
Glutamic acid	1.083	73
Proline	0.304	22
Glycine	0.404	27
Alanine	0.377	27
Valine	0.402	28
Methionine	0.079	6
Isoleucine	0.418	29
Leucine	0.858	61
Tyrosine	0.209	14
Phenylalanine	0.137	9
Tryptophan	0.143[b]	10
Cysteine	0.084[c]	6
	Total Residues	472

[a] Obtained by averaging the values for 24-, 48-, and 72-h hydrolyses except as follows: serine and threonine are least-squares extrapolations to zero hydrolysis time. Alanine, valine, and isoleucine are from the 72-h hydrolysis values. [b] Based on a tryptophan content of 10 residues per mole of enzyme as determined by the method of Goodwin and Morton (1946). [c] Average of three determinations as cysteic acid after 24-h hydrolysis (Hirs, 1956).

for each residue, in terms of micromoles per milligram of protein, is listed in the second column and, with the exception of tryptophan, represents the results obtained from the hydrolysis of three separate protein samples. The values shown in the last column depict the amino acids (rounded off to the nearest whole number) per 57 000 g of enzyme. From the data presented, a partial specific volume ($\bar{v}$) of 0.733 ml/g of protein can be calculated (Cohn and Edsall, 1943).

DPGM's estimated extinction coefficient of 1.65 cm^2/mg (see Experimental Procedures) is in good agreement with the value expected from the enzyme's tyrosine and tryptophan content (Edelhoch, 1967).

Discussion

Until now, no simple scheme has been described for the preparation of large quantities of purified erythrocyte diphosphoglycerate mutase. As pointed out in the text, the previous purification efforts of Rose and Whalen (1973) and Sasaki et al. (1975) resort to the use of isoelectric focusing in order to obtain DPGM free of extrinsic MPGM. In retrospect, however, isoelectric focusing appears to be critical only with regard to the latter procedure. Thus, Rose and Whalen seem to have accomplished the isolation of a small quantity of purified DPGM without the necessity of employing electrophoretic techniques, but because of the lack of experimental evidence, it has been difficult to ascertain whether their procedure yields homogeneous enzyme. In fact, from a consideration of specific activities, it appears that the aforementioned investigators achieved only a 60% purification of diphosphoglycerate mutase. This is brought more clearly into focus in the following discussion.

Upon cursory examination, the percent MPGM activity (20% based on dimutase) associated with our purified enzyme appears to be higher than the 1–3% previously reported by Rose (1968), and Rose and Whalen (1973). This discrepancy, however, is due only to a difference in the methods employed for reporting specific activities. Thus, Rose (1968) recommends that under assay conditions comparable to ours, the observed rates for DPGM should be multiplied by 3 in order to obtain a true rate of product formation.[4] When this is done, we obtain a specific activity for DPGM of 16.8 U/mg instead of the 5.6 U/mg reported in Table I. Applying the same principles, the highest specific activity for the dimutase obtained by Rose is 10.0 U/mg. When the intrinsic MPGM activity of our purified enzyme is determined, using the method employed by Rose (Cowgill and Pizer, 1956), we obtain a specific activity of 0.06 U/mg. This value represents 0.36% of the activity of the dimutase and is 2–8 times less than previously published values (Rose, 1968; Rose and Whalen, 1973).

Of prime importance is the finding that homogeneous

[4] We have determined that there is no difference in rates when DPGM is assayed either under our conditions or the conditions specified by Rose (1968).

DPGM intrinsically manifests both MPGM and DPGP activities. Definitive evidence for the multifunctionality of the DPGM molecule is obtained from the thermal inactivation data presented in Figure 2. The stimulation by various anions of the 2,3-DPG phosphatase activity of DPGM (Table II) also lends strong support to the variegated catalytic capacity of the DPGM enzyme. At this point, it is interesting to note that Rose (1973) has observed that erythrocyte diphosphoglycerate mutase activity is inhibited by several of the very ions that cause 2,3-DPG phosphatase activation. Thus, it seems reasonable to postulate that the intracellular concentrations of certain anions may be the key to the regulation of red cell 2,3-DPG levels. Important to the above hypothesis is the recent observation (Hass and Miller, 1975) that the intrinsic DPGP activity of diphosphoglycerate mutase is sufficient to account for all of the 2,3-DPG phosphatase activity of the red cell (Rose and Liebowitz, 1970).

As already mentioned, one of the benefits of our purification scheme is that a distinct, homogeneous monophosphoglycerate mutase can be obtained from the same batch of erythrocytes used to isolate DPGM (Kappel et al., 1975; R. H. Sheibley and L. F. Hass, manuscript in preparation). This has allowed us to determine the total catalytic capacities of both mutases within the red cell and, with knowledge of specific activities, we have found that the intracellular concentration of DPGM (~133 μg/ml packed cells) exceeds that of MPGM (~13.9 μg/ml of packed cells) by approximately an order of magnitude (Hass and Miller, 1975).

Purified MPGM apparently has a native molecular weight and a subunit composition similar to that of DPGM. Moreover, the 2,3-DPG phosphatase activity of both enzymes is greatly stimulated in the presence of glycolate-2-P. Despite these and other similarities, each protein possesses distinct molecular characteristics (e.g., differences in thermal stability as shown in Figure 2) which could provide valuable information with regard to enzyme structure and function.

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