Partial purification and characterization of human erythrocyte phosphoglycollate phosphatase

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Human erythrocytes contain a phosphatase that is highly specific for phosphoglycollate. It shows optimum pH of 6.7 and has K_m 1 mM for phosphoglycollate. The molecular weight appears to be about 72 000. The enzyme is a dimeric molecule having subunits of mol.wt. about 35 000. It could be purified approx. 4000-fold up to a specific activity of 5.98 units/mg of protein. The activity of the enzyme is Mg^{2+} -dependent. Co^{2+} , and to a smaller extent Mn^{2+} , may substitute for Mg^{2+} . Half-maximum inhibition of the phosphatase by 5,5'-dithiobis-(2-nitrobenzoate), EDTA and NaF is obtained at 0.5 μM , 1 mM and 4 mM respectively. Moreover, it needs a univalent cation for optimum activity. Phosphoglycollate phosphatase is a cytoplasmic enzyme. Approx. 5% of its total activity is membrane-associated. This part of activity can be approx. 70% solubilized by freezing, thawing and treatment with 0.25% Triton X-100.

Phosphoglycollate phosphatase (EC 3.1.3.18) is present in a variety of plants (Randall *et al.*, 1971). Richardson & Tolbert (1961) were the first to find a phosphatase activity specific for phosphoglycollate in tobacco leaves. Later Badwey (1977) demonstrated the existence of such an enzyme in human erythrocytes. Barker & Hopkinson (1978) have since detected phosphoglycollate phosphatase and its isoenzymes in all human tissues, where they occur with different amounts of activity.

The function of phosphoglycollate phosphatase is still unknown. Suggestions that it may regulate the normal concentration of phosphoglycollate (Badwey, 1977), which might be present in human erythrocytes at low steady-state concentrations, cannot be excluded, although it is still questionable whether phosphoglycollate is present in human erythrocytes (Örström, 1951; Richardson & Tolbert, 1961).

In view of the activating effect of phosphoglycollate on 2,3-bisphosphoglycerate phosphatase activity of the bifunctional enzyme 1,3-bisphosphoglycerate mutase/2,3-bisphosphoglycerate phosphatase (EC 2.7.5.4) (Rose & Liebowitz, 1970; Rose, 1970, 1976), phosphoglycollate phosphatase is of especial interest, since 2,3-bisphosphoglycerate binds to human haemoglobin and alters its oxygen affinity

(Chanutin & Curnish, 1967; Ponce *et al.*, 1971; Hamasaki & Rose, 1974).

The present paper describes the partial purification and characterization of phosphoglycollate phosphatase from human erythrocytes.

Experimental

Materials

Phosphoglycollate, D(+)-2-phosphoglycerate, D(-)-3-phosphoglycerate, phosphoenolpyruvate, phosphoglycerate mutase (rabbit muscle), enolase (yeast) and 2,3-bisphosphoglycerate were purchased from Sigma, München, W. Germany. All other phospho compounds were from Boehringer, Mannheim, W. Germany, except β -glycerophosphate, which was from Merck, Darmstadt, W. Germany, and D-fructose 1,6-bisphosphate, glucose 1-phosphate and glucose 6-phosphate, which were from Serva, Heidelberg, W. Germany. Mops (4-morpholinepropanesulphonic acid) was from Paesel, Frankfurt, W. Germany, and di-isopropyl phosphorofluoridate from Fluka, Buchs, Switzerland. Triton X-100, Tween 20, Brij 30, SDS and the thiol-specific reagents were from Serva, and potassium deoxycholate was from Merck. All other chemicals used were of analytical grade and were obtained from Serva or Merck. All metal

Abbreviation used: SDS, sodium dodecyl sulphate.

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ions were used as chlorides, and the anions as sodium salts. Sephadex G-200 and DEAE-Sephacrose CL-6B were products of Pharmacia, Uppsala, Sweden. Ultrogel Aca44 was from LKB, München, W. Germany. Myoglobin (horse), lactate dehydrogenase (pig heart) and pyruvate kinase (rabbit muscle) were from Serva. Haemoglobin (human) was from Sigma. Trypsin inhibitor, bovine serum albumin and RNA polymerase for SDS/polyacrylamide-gel electrophoresis were obtained from Boehringer. Crystalline bovine serum albumin as a standard for protein determination was from Biomol, Ilvesheim, W. Germany. Fresh human blood of the O Rh⁺ (Rhesus-positive) group was obtained from Deutsches Rotes Kreuz, Bad Kreuznach, W. Germany. Water was double-distilled in a quartz apparatus and used for preparation of all solutions.

Enzyme assay

Phosphatase activity was determined by the method of Fiske & SubbaRow (1925), as modified by Lacy (1965), in an automated discontinuous assay (Wolf, 1972). The test solution contained 50 mM-Mops buffer, pH 6.7, 200 mM-KCl, 5 mM-MgCl₂, 5 mM-phosphoglycollate and various concentrations of protein in a final volume of 5.0 ml.

The pH-dependence was measured with, as the buffers, acetic acid (pH 4.2–4.7), Tris/maleate (pH 5.1–6.3), Mops (pH 6.7–7.1) and Tris (pH 7.4–9.6), at a concentration of 100 mM (Tris/maleate, 50 mM). Either HCl or KOH was used to adjust the buffers to the desired pH. The maximal drift in pH during the experiment was 0.1 unit. Phosphoglycollate and *p*-nitrophenyl phosphate were used as substrates, each at the same concentration of 5 mM. The phosphohydrolase properties of phosphoglycollate phosphatase were tested towards a variety of phospho compounds at a concentration of 2 mM. EDTA concentration was varied at optimum concentration of Mg²⁺, the incubation time being 10 min. Other bivalent cations such as Ca²⁺, Sr²⁺, Ba²⁺, Mn²⁺, Ni²⁺, Co²⁺, Zn²⁺ and Cd²⁺ were investigated as substitutes for Mg²⁺ at a concentration of 1 mM. Before measurement of the activity, 3 ml of a fraction containing partially purified enzyme was diluted to 15 mM with respect to EDTA and dialysed for 18 h against 5 litres of a solution containing 10 mM-mercaptoethanol, 100 mM-KCl and 5 mM-Mops, pH 7.1. Thereafter the EDTA concentration was again adjusted to 15 mM.

The activity at optimum concentration of K⁺ was determined by variation of K⁺ concentration. The activity at this optimum concentration was compared with the activity of the enzyme at the same concentration of Li⁺, Na⁺, Rb⁺, Cs⁺ and NH₄⁺.

F⁻, Cl⁻, Br⁻, I⁻, NO₂⁻ and CH₃-CO₂⁻ were tested for their influence on enzyme activity and

the influence of thiol-group-specific reagents on activity was examined. Before measurement of the activity by adding 5 mM-phosphoglycollate at pH 7.1, the enzyme was incubated for 1 h at 37°C in the test solution in the presence of different inhibitor concentrations at the same pH value. Mercaptoethanol had been removed by dialysis of 2 ml of the partially purified enzyme fraction for 2 h against 5 litres of a solution containing 100 mM-KCl, 5 mM-MgCl₂ and 50 mM-Mops buffer, pH 7.1. The assay mixtures contained 1.5 µg of protein.

Phosphoglycerate phosphomutase (EC 5.4.2.1) activity was determined by the method of Grisolia (1962).

The temperature was maintained at 37°C. One unit of activity is defined as the liberation of 1 µmol of P_i/min under the standard conditions listed above, and specific activity is expressed in units/mg of protein.

Gel electrophoresis

Staining for protein with Coomassie Brilliant Blue and destaining was by the procedure of Fairbanks *et al.* (1971). Analytical disc electrophoresis was performed in diethyl barbituric acid/Tris buffer, pH 7.0, with 0.7 cm × 8 cm polyacrylamide gels (5% acrylamide) at 4 mA/tube for 70 min. Phosphoglycollate phosphatase activity was located by the method of Allen & Hyncik (1963) with phosphoglycollate as substrate. All other conditions were as described above under 'Enzyme assay'.

Disc electrophoresis in the presence of SDS was performed in Tris/glycine buffer, pH 8.9, with 0.7 cm × 8 cm polyacrylamide gels (7.5% acrylamide) at 4 mA/tube for 70 min. The method of Weber & Osborn (1969) was used for determination of subunits of the enzyme by SDS/polyacrylamide-gel disc electrophoresis. Marker proteins were trypsin inhibitor (mol.wt. 21 500), bovine serum albumin (mol.wt. 68 000) and β- and γ-subunits of RNA polymerase (mol.wts. 155 000 and 165 000). Analytical polyacrylamide gels were loaded with 9 µg of protein/gel and the SDS/polyacrylamide gels with 45 µg.

Determination of molecular weight

The molecular weight of the enzyme was calculated after determination of the sedimentation coefficient by using the ultracentrifugation activity test (Bodmann *et al.*, 1960), assuming a partial specific volume of 0.73. In addition, the native enzyme was subjected to molecular-exclusion chromatography on a Sephadex G-200 column (1.5 cm × 81 cm) equilibrated with the same buffer as that mentioned below under 'Preparation and partial purification of enzyme' but in the absence of mercaptoethanol. Marker proteins were myoglobin

(mol.wt. 17800), haemoglobin (mol.wt. 64 500), lactate dehydrogenase (mol.wt. 130000) and pyruvate kinase (mol.wt. 230000). Dextran Blue was used for determination of V_0 . Fractions of volume 3.4 ml were collected. Detection of enzyme was based on measurement of enzyme activity as described above. Protein and dextran were detected by using a spectrophotometer as described by Andrews (1965). A constant flow rate of 9 ml/h was maintained by a peristaltic pump.

Protein determination

Protein was determined in the presence of mercaptoethanol by a modified Lowry method with chloramine-T as an oxidizing agent (Higuchi & Yoshida, 1977).

Preparation and partial purification of enzyme

All operations were performed at 0–4°C. Human erythrocytes, which had been in contact with ACD-AG stabilizer (sodium citrate, citric acid, glucose, adenine and guanosine) for less than 3 days, were thoroughly washed three times with 0.155 M-KCl (with centrifugation at 2500g for 20 min) to remove leucocytes and fat. The washed and packed cells (350 ml) were lysed in a solution containing 0.04 mM-ZnCl₂, 5 mM-MgCl₂, 10 mM-mercaptoethanol, 0.1 mM-di-isopropyl phosphorofluoridate and 10 mM-Mops buffer, pH 7.2 (final concentrations). The final volume was 1400 ml and the final pH 6.9. After centrifugation at 20000g for 1 h, the supernatant was decanted. Buffer solution containing 0.04 mM-ZnCl₂, 5 mM-MgCl₂, 10 mM-mercaptoethanol, 0.1 mM-di-isopropyl phosphorofluoridate, 20 mM-KCl and 10 mM-Mops buffer, pH 7.1, was added to the sediment to yield the original volume of 1400 ml. This suspension was again centrifuged under the above conditions. The last step was repeated. The three supernatants were combined and made 55% saturated with respect to (NH₄)₂SO₄ by adding solid (NH₄)₂SO₄. The pH decreased to 6.6–6.8, but was not re-adjusted to pH 7.1. The mixture was left for 3 h, then it was centrifuged at 11500g for 30 min and the precipitate dissolved in 200 ml of the buffer solution. (NH₄)₂SO₄ was added to give 25% saturation and after 2 h the solution was centrifuged at 9800g for 30 min. The (NH₄)₂SO₄ concentration of the supernatant was increased to 38% saturation and the mixture was centrifuged at 47000g after 3 h for 20 min. The precipitate was taken up in buffer solution. After dialysis (2 h against 2 litres of buffer solution) the 38%-satd.-(NH₄)₂SO₄ fraction was clarified by centrifugation at 14000g for 10 min. It was stored at –20°C or immediately used for chromatography.

The 38%-satd.-(NH₄)₂SO₄ fraction was applied to a column (1.45 cm × 25 cm) of DEAE-Sephacel CL-6B equilibrated with buffer solution, pH 7.1. The

column was then washed with the same buffer (125 ml) and the enzyme was eluted by a linear gradient of 20–500 mM-KCl in buffer, pH 6.8, containing 0.04 mM-ZnCl₂, 5 mM-MgCl₂, 10 mM-mercaptoethanol, 0.1 mM-di-isopropyl phosphorofluoridate and 10 mM-Mops (500 ml of each starting buffer in the reservoirs). Fractions of volume 17.5 ml were collected at a flow rate of 36 ml/h. Fractions 15–20, which contained most of the enzyme activity, as monitored by the enzyme assay, were pooled, dialysed against starting buffer (2 litres, for 2 h) and rechromatographed under the above conditions. Fractions 17 and 18 of the rechromatography were made 57% saturated with (NH₄)₂SO₄ and left for 2 h. The precipitate obtained after centrifugation at 23000g for 20 min was suspended in 4 ml of the subsequent solution: 0.04 mM-ZnCl₂, 5 mM-MgCl₂, 10 mM-mercaptoethanol, 100 mM-KCl, 0.1 mM-di-isopropyl phosphorofluoridate and 20 mM-Mops buffer, pH 7.2. It was clarified by centrifugation at 14000g for 10 min and applied to a column (2 cm × 108 cm) of Ultrogel AcA44 equilibrated with the same buffer. Fractions of volume 9 ml were collected at a flow rate of 18 ml/h. The protein obtained from the active fractions of molecular-exclusion chromatography was subjected to polyacrylamide-gel electrophoresis.

Solubilization experiments

These were performed to compare the amounts of cytoplasmic and membrane-associated enzyme activity. For this purpose 350 ml of packed erythrocytes was diluted 1:1 with a solution giving final concentrations as described above (0.04 mM-ZnCl₂, 5 mM-MgCl₂, 10 mM-mercaptoethanol, 0.1 mM-di-isopropyl phosphorofluoridate and 10 mM-Mops buffer, pH 7.1). Then complete haemolysis was performed by freezing (at –80°C for 40 min) and thawing. The washing procedure was as mentioned above, except that a fourth centrifugation followed. The sediment was frozen at –80°C in small portions and stored at –20°C.

The part of the enzyme solubilized by freezing and thawing of the membranes was removed by centrifugation (10 ml for each assay) at 17000g for 30 min before solubilization by detergents. The sediment was suspended in 5 ml of buffer solution (final volume) containing various concentrations of the detergents. The mixture was stirred vigorously and left for 10 min. After centrifugation under the above conditions the membranes were resuspended in 5 ml (final volume) of buffer solution. Enzymic activity was measured as described above. The detergents investigated for their solubilizing efficiency were Triton X-100, Tween 20, SDS, potassium deoxycholate and Brij 30.

For molecular-exclusion chromatography of the solubilized phosphoglycollate phosphatase, the

applied solution was prepared as follows: 162 ml of sediment was suspended in a final volume of 264 ml containing buffer solution and 0.25% Triton X-100. After centrifugation as described above, the supernatant was made saturated with 30% $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 98 000 *g* for 1 h. The supernatant then was made 60% saturated with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 230 000 *g* for 1 h. The dark-brown pellet was treated as described for the 57%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut of fractions 17 and 18 of rechromatography.

Results

Phosphoglycollate phosphatase shows an activity of about 300–350 units/kg of packed erythrocytes.

Applied to a DEAE-Sephacrose CL-6B column, the enzyme is eluted by a linear gradient of KCl in the range 50–150 mM, with the maximum activity appearing at 110 mM (Fig. 1). After molecular-exclusion chromatography the enzyme appears in the effluent as a single sharp peak. The most active fraction has a specific activity of 5.98 units/mg and the enzyme is purified 4016-fold (Table 1).

When the 38%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut (see Table 1) is subjected to molecular-exclusion chromatography, a second peak emerges beside the phosphoglycollate phosphatase peak; this is due to acid phosphatase (Fisher & Harris, 1971). The most active fraction of the phosphoglycollate phosphatase peak has a specific activity of approx. 0.9–1.0 unit/mg, corresponding to a purification of 750–1000-fold (Fig. 2a).

The same elution profile is obtained when the enzyme is applied to the column after removal of the cytoplasmic fraction by washing and treatment of the membranes with Triton X-100. Additionally, a third peak, also showing phosphoglycollate phosphatase activity, appears before the first one (Fig. 2b).

The acid phosphatase does not attack phosphoglycollate (see Fig. 2b).

The erythrocyte membranes, treated by freezing at -80°C , thawing and washing four times, still have phosphoglycollate phosphatase as well as acid phosphatase activity (see Fig. 2b). The mem-

Fig. 1. Ion-exchange chromatography of the 38%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut on DEAE-Sephacrose CL-6B

The phosphoglycollate phosphatase rich fraction obtained by precipitation in 38%-satd. $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 20 ml of starting buffer, pH 7.1 (see the Experimental section), applied to a DEAE-Sephacrose CL-6B column (1.45 cm $\times$ 25 cm) and eluted by a linear gradient of 20–500 mM-KCl in buffer, pH 6.8. Fractions of volume 17.5 ml were collected at a flow rate of 36 ml/h. Fractions 15–20, which contained most of the enzyme activity, were pooled. —, Salt gradient ([KCl]); ●, phosphatase activity; ○, protein (A_{280}).

Table 1. Partial purification of phosphoglycollate phosphatase
For experimental details see the text.

Preparation step	Volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
Packed cells	350			
Haemolysate	2450	97970	124	0.00127
55%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut	220			
25%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut	230	2550	90.4	0.035
38%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut	20	506	90.4	0.179
Fractions 15–20 from DEAE-Sephacrose CL-6B chromatography	105	41.4	41.1	0.994
Dialysis of fractions 15–20	105	41.4	41.1	0.994
Fractions 17 + 18 of rechromatography	35	20	26.4	1.32
57%-satd. $(\text{NH}_4)_2\text{SO}_4$ cut	4	6.9	13.7	1.97
Fraction 23 of molecular-exclusion chromatography	9	0.85	5.08	5.98

Fig. 2. Molecular-exclusion chromatography on Ultrogel Aca 44 of the 38%-satd.-(NH₄)₂SO₄ cut (a) and of the membrane-associated solubilized activity (b)

(a) A 2.5 ml portion of the 38%-satd.-(NH₄)₂SO₄ cut was applied to the column (2 cm × 108 cm) and eluted with equilibration solution. Fractions of volume 9 ml were collected at a flow rate of 18 ml/h. Fractions 17 and 18 of rechromatography on DEAE-Sephacrose CL-6B were made 57% saturated with (NH₄)₂SO₄. The precipitate was suspended in 4 ml of equilibration solution and further treated as above. The elution profile of activity is the same as above, but no longer shows the acid phosphatase activity. (b) After removal of the cytoplasmic fraction by washing, the membranes were mixed with buffer solution (0.04 mM-ZnCl₂, 5 mM-MgCl₂, 10 mM-Mops buffer, pH 7.1, 10 mM-mercaptoethanol, 0.1 mM-di-isopropyl phosphofluoridate and 20 mM-KCl) containing 0.25% Triton X-100 (see the Experimental section). The solubilized material was subjected to two (NH₄)₂SO₄ precipitations (30% and 60% saturation) and thereafter treated as described for the 38%-satd.-(NH₄)₂SO₄ cut. Total

brane-associated phosphoglycolate phosphatase activity is about 5% of the total activity. This value can be lowered to approx. 2.5% by repeated freezing and thawing and one centrifugation. After resuspension of the membranes in the presence of 0.25% Triton X-100, the remaining activity is less than 2% of the total. It corresponds to 30% of the membrane-associated activity.

Triton X-100 can solubilize about 25% of the membrane-associated activity at 0.25% detergent concentration. Brij 30 solubilizes only 7% of the active enzyme (at a detergent concentration of 0.2%). Tween 20 solubilizes only 5% (at 0.5%), whereas SDS and deoxycholate show no effect on solubilization at 0.02 and 0.045% detergent concentration respectively. The use of higher detergent concentrations causes increasing denaturation of the active enzyme.

The fraction of highest specific activity after molecular-exclusion chromatography shows one main band after analytical polyacrylamide-gel

Fig. 3. Augustinsson-Hofstee plot for the determination of the K_m value of partially purified phosphoglycolate phosphatase

The assay mixtures contained 50 mM-Mops buffer, pH 6.7, 200 mM-KCl, 5 mM-MgCl₂ and various concentrations of phosphoglycolate in a final volume of 5.0 ml; the temperature was 37°C. For further details see the Experimental section. Five measurements were carried out at each concentration. Bars indicate $\pm$ S.E.M.

protein cannot be shown because of the Triton X-100 still present in the second precipitate. ●, Phosphoglycolate phosphatase activity; ▼, acid phosphatase activity; ■, acid phosphatase activity, measured with phosphoglycolate as substrate; ○, protein (A₂₈₀).

electrophoresis. This band coincides with the band stained for activity. When the material is subjected to SDS/polyacrylamide-gel electrophoresis the material in the band is split into apparently identical subunits with a mol.wt. of about 35000. The dimer has an apparent mol.wt. of 90000 determined by molecular-exclusion chromatography on Sephadex G-200, and of 72000 by determination of the sedimentation coefficient.

The Michaelis constant of the partially purified enzyme was 1mM (Fig. 3). The dependence of reaction rate on protein concentration is linear. Partially purified phosphoglycollate phosphatase has maximal activity at pH6.7 with phosphoglycollate as substrate and at pH5.6 with *p*-nitrophenyl phosphate as substrate. Thus phosphoglycollate phosphatase appears to have acid phosphatase activity (Fig. 4).

Partially purified phosphoglycollate phosphatase is a highly specific phosphatase for phosphoglycollate, as shown in Table 2, where various phospho compounds and their reaction rates expressed as percentages of observed activity towards phosphoglycollate are listed. Among the mono-, di- and tri-

nucleotides, only ADP and ATP are weakly hydrolysed. Very little activity was observed towards D-fructose 1,6-bisphosphate, α -glycerophosphate and β -glycerophosphate.

Phosphoglycollate phosphatase is strongly inhibited by EDTA. Half-maximum inhibition occurs at 1mM after 10min incubation. Mg^{2+} seems to be bound very tightly to the enzyme, because total abolition of activity needs 20mM-EDTA in the test solution. Dialysis of the enzyme against 10mM-EDTA or addition of EDTA to the enzyme solution to 15mM concentration before measurement of the activity leads to 25 and 15% remaining activity respectively.

Total loss of activity, which can be restored to 90% by adding back Mg^{2+} , is obtained also by the procedure described in the Experimental section. The activity optimum was found for Mg^{2+} at 0.4mM.

Not only Mg^{2+} but other bivalent cations also activate phosphoglycollate phosphatase (see Table 3).

Fig. 4. pH-dependence of partially purified phosphoglycollate phosphatase

Assay conditions: 100mM of the following buffers were used according to their optimal buffer ranges: acetic acid (4.2–4.7), Tris/maleate (5.1–6.3), Mops (6.7–7.1) and Tris (7.4–9.6). The other conditions were the same as described in Fig. 3, except that phosphoglycollate or *p*-nitrophenyl phosphate was present in a concentration of 5mM. ●, pH-activity profile with phosphoglycollate as substrate; ○, pH-activity profile with *p*-nitrophenyl phosphate as substrate.

Table 2. Phosphohydrolase properties of partially purified phosphoglycollate phosphatase

The Table shows the phosphohydrolase rate towards various phospho compounds relative to that towards phosphoglycollate as substrate. The final concentration of the phospho compounds was 2mM in all cases. Assay conditions were the same as in Fig. 3, except that the substrate was varied. For experimental details see the text.

Substrate (2mM)	Activity (%)
Phosphoglycollate	100
<i>p</i> -Nitrophenyl phosphate	22.3
β -Glycerophosphate	3.9
DL- α -Glycerophosphate	2.6
D-Fructose 1,6-bisphosphate	1.1
Fructose 6-phosphate	0
Glucose 1-phosphate	0
Glucose 6-phosphate	0
Phosphoenolpyruvate	0
D(+)-2-Phosphoglycerate	0
D(-)-3-Phosphoglycerate	0
2,3-Bisphosphoglycerate	0
Cyclic AMP	0
AMP	0
ADP	1.1
ATP	1.6
CMP	0
CDP	0
CTP	0
GMP	0
GDP	0
GTP	0
IMP	0
UMP	0
UDP	0

Table 3. Influence of bivalent cations (Me^{2+}) on the activity of partially purified phosphoglycollate phosphatase

The assay mixtures contained 5 mM-Mops buffer, pH 6.7, 200 mM-KCl, 5 mM-phosphoglycollate and 1 mM of the bivalent ion (chloride salt). Mg^{2+} had been removed by dialysis and the addition of EDTA to zero activity. For experimental details see the text.

Metal ion (1 mM)	$v_{[Me^{2+}]} / v_{[Mg^{2+}]}$
Mg^{2+}	1.00
Co^{2+}	0.95
Mn^{2+}	0.64
Ni^{2+}	0.32
Ba^{2+}	0.30
Sr^{2+}	0.24
Ca^{2+}	0.03
Cd^{2+}	0
Zn^{2+}	0

Table 4. Effect of various anions on the activity of partially purified phosphoglycollate phosphatase

The assay mixture contained 5 mM-Mops buffer, pH 6.7, 5 mM- $MgCl_2$, 5 mM-phosphoglycollate and 100 mM of the anion (A^-) (sodium salt). For experimental details see the text.

Anion (100 mM)	$v_{[A^-]} / v_{[Cl^-]}$
F^-	0
Cl^-	1.00
Br^-	2.17
I^-	1.13
NO_3^-	1.20
$CH_3CO_2^-$	0.35

Phosphoglycollate phosphatase needs K^+ for optimal activation (100 mM). The ion can be replaced by Li^+ , Na^+ , Rb^+ , Cs^+ and NH_4^+ without any significant loss of activity. The effects of anions on the enzyme are shown in Table 4.

The activity of phosphoglycollate phosphatase is remarkably affected by thiol-group-specific reagents. The enzyme activity is 50% inhibited by 5,5'-dithio-bis-(2-nitrobenzoate), *p*-hydroxymercuribenzoate and *o*-iodosobenzoate at 0.5 μ M, 2 μ M and 10 μ M respectively (see Fig. 5).

Partially purified phosphoglycollate phosphatase shows no phosphoglycerate phosphomutase activity.

After approx. 150-fold purification (see Table 1), the enzyme can be stored at $-20^\circ C$ for 4 weeks without significant loss of activity. After DEAE-Sepharose CL-6B chromatography or any further step of purification, the enzyme is fully deactivated by storage at $-20^\circ C$ within a few hours. In contrast,

Fig. 5. Inhibition of partially purified phosphoglycollate phosphatase by thiol-specific reagents

The assay mixtures containing 1.5 μ g of protein, 50 mM-Mops buffer, pH 7.1, 5 mM- $MgCl_2$, 200 mM-KCl and various concentrations of the thiol-specific reagents were incubated for 60 min at $37^\circ C$. Thereafter the activity was measured by adding 1 ml of 25 mM-phosphoglycollate to each assay mixture (5.0 ml final volume). Mercaptoethanol had been removed by dialysis. ●, Inhibition by 5,5'-dithio-bis-(2-nitrobenzoate); ▼, inhibition by *o*-iodosobenzoate; ■, inhibition by *p*-hydroxymercuribenzoate; ○, no inhibitor.

activity remains without significant loss for a few days when the enzyme is stored at $4^\circ C$.

Discussion

The existence of phosphoglycollate phosphatase in human erythrocytes (Badwey, 1977; Barker & Hopkinson, 1978) has been confirmed and the prediction of Barker & Hopkinson (1978), that the enzyme should be a dimeric molecule, verified.

The fact that the mol.wt. of 90 000 determined by molecular-exclusion chromatography is higher than that obtained by ultracentrifugation activity-test experiments may be due to asymmetry of the molecule.

It is still an open question whether the second peak of phosphoglycollate phosphatase activity found in molecular-exclusion chromatography represents a larger aggregate of the enzyme or whether the corresponding molecule is another distinct enzyme that is entirely membrane-bound.

Phosphoglycollate phosphatase is a cytoplasmic enzyme. About 5% of the total activity is obviously

only membrane-associated, not membrane-bound as an intrinsic protein, although solubilizing agents such as Triton X-100 are necessary to lower this portion of activity to approx. 30% of the total membrane-associated activity.

These facts reopen the discussion about the existence of a K^+ -activated membrane-bound *p*-nitrophenyl phosphatase present in human erythrocytes, being identical in part or totally (Rega *et al.*, 1973a,b, 1974) with the high-affinity Ca^{2+} -ATPase isolated by Wolf *et al.* (1977). From the following similarities the observed *p*-nitrophenyl phosphatase is identical with the phosphoglycollate phosphatase isolated in the present work: (1) both are Mg^{2+} - and K^+ -dependent; (2) they can be activated by Na^+ ; (3) they hydrolyse *p*-nitrophenyl phosphate; (4) their membrane-associated amounts of activity are comparable. In contrast, the possibility of the identity of phosphoglycollate phosphatase with Ca^{2+} -ATPase can be excluded, not at the least on account of their different mol.wts.: 145 000 for the active subunit of the Ca^{2+} -ATPase (Wolf *et al.*, 1977) and 72 000 for the active molecule of phosphoglycollate phosphatase (subunit 35 000).

Phosphoglycollate phosphatase shows a high specificity towards phosphoglycollate. This is in marked contrast with acid phosphatase, which does not hydrolyse phosphoglycollate, but does attack α -glycerophosphate to a considerable extent (Tsuboi & Hudson, 1953; Luffman & Harris, 1967). Additionally, its mol.wt. of 14 800 (Fisher & Harris, 1971) indicates that the enzymes are not identical. Although the acid phosphatase tends to form dimers and tetramers (Kaczmarek, 1976), it can be excluded, on account of their different molecular weights, that the enzymes are identical.

Besides Mg^{2+} , Co^{2+} and to a smaller extent Mn^{2+} also activate phosphoglycollate phosphatase, whereas ions such as Cd^{2+} and Zn^{2+} fully deactivate the enzyme. These findings are similar to the results obtained by Tsuboi & Hudson (1955) for human erythrocyte acid phosphatase. Like acid phosphatase (Tsuboi & Hudson, 1955), phosphoglycollate phosphatase is strongly inhibited by thiol-specific reagents. Therefore thiol groups obviously are essential for activity in both cases.

The similar results for different univalent cations may be due to a specific chloride effect rather than to a non-specific ionic-strength effect, because the anions tested show both inhibition and activation of activity.

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