Purification and Characterization of a High Molecular Weight Type 1 Phosphoprotein Phosphatase from the Human Erythrocyte*

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The major Mn**-activated phosphoprotein phosphatase of the human erythrocyte has been purified to homogeneity from the cell hemolysate. It is sensitive to both inhibitors 1 and 2 of rabbit skeletal muscle, preferentially dephosphorylates the β subunit of the phosphorylase kinase, and dephosphorylates a broad range of substrates including phosphorylase a, p-nitrophenyl phosphate, phosphocasein, the regulatory subunit of cyclic AMP-dependent protein kinase, and both spectrin ($K_\text{a} = 10 \text{ mM}$) and pyruvate kinase ($K_\text{a} = 18 \text{ mM}$) purified from the human erythrocyte. The purified enzyme is stimulated by Mn** and to a lesser extent by higher concentrations of Mg**.

The purification procedure was selected to avoid any change in molecular weight, hence subunit composition, between the crude and purified enzyme. Maintenance of the original structure is demonstrated by non-denaturing gel electrophoresis and gel filtration chromatography. Gel filtration of the purified holoenzyme shows a single active component with a Stokes radius of 58 Å at a molecular weight position of 180,000. Sedimentation velocity in a glycerol gradient gives a value of 6.1 for $s_{20,w}$. Together these data indicate a molecular weight of about 155,000. Two bands of equal intensity appear on sodium dodecyl sulfate-gel electrophoresis at molecular weights of 61,700 and 36,300, suggesting a subunit composition of two 36,000 and one 62,000 subunits.

The 36-kDa catalytic subunit can be isolated by freezing and thawing the holoenzyme or by hydrophobic chromatography of the holoenzyme. The catalytic subunit shows unchanged substrate and inhibitor specificity but altered metal ion activation.

It has been shown that several cytosolic proteins in the human erythrocyte can be phosphorylated either in vitro or in the intact cell (1, 2) and we have proposed that this may be a mechanism for regulating pyruvate kinase activity in vivo (2). There are also many reports of phosphorylation of the membrane proteins of the cell but the function of these reactions is still not clear (3). If phosphorylation is a mechanism for the regulation of either the function or metabolism of the erythrocyte, there must be a mechanism for reversing the phosphorylation of these proteins.

Preliminary reports have described cytosolic phosphoprotein phosphatase activity capable of dephosphorylating spectrin (4, 5) as well as pyruvate kinase (6). A membrane-bound phosphoprotein phosphatase, capable of dephosphorylating membrane-associated proteins has been described by Fischer and co-workers (7). The relationship among these activities is not known.

Usui and co-workers (8) have found several forms of phosphoprotein phosphatase in human erythrocyte cytosol. The phosphatases differ in their activity toward various protein substrates and in their molecular weight. Usui and co-workers have purified 3000-fold, to apparent homogeneity, a 104 kDa phosphatase from the erythrocyte. That enzyme differs in every measured characteristic from the enzyme to be described here (see "Discussion").

Multiple forms of phosphatase activity have been purified from other tissues but it is not clear whether all the forms exist in the tissues or result from breakdown during the purification procedure. In many cases an approximately 30,000-Da subunit can be generated from a larger species by treating the enzyme with ethanol, aceton, or trypsin, or by freezing (9). This appears to be a catalytic subunit common to many of the phosphoprotein phosphatases (9). In the red blood cell, 3 of the 4 reported phosphatases can be converted to an active subunit of 35,000 Da. However, it remains unclear what the relationship is between this catalytic subunit and the noncatalytic peptides associated with the more complex forms of the enzyme. Several reports have suggested that the noncatalytic peptides of phosphoprotein phosphatases function as inhibitors of the enzyme (10, 11) and that appears well established in at least one case (12). However, the extent of purification has made it uncertain as to whether the inhibitors were truly subunits of the phosphatase or co-purifying proteins.

In this paper we describe the purification and properties of the major Mn**-activated phosphoprotein phosphatase in the human erythrocyte which is also the highest molecular weight phosphatase in that cell. The purification procedures reported here were developed to maintain the molecular weight form of the enzyme found in the initial hemolysate as a prerequisite for more detailed studies on the subunit structure and functions.

MATERIALS AND METHODS

Ultraloe AcA 34 and Ampholine PAG plates were obtained from LKB, Sephadex G-200, DEAE A-50, and Sephacryl were obtained from Pharmacia P-L Biochemicals, and CM Bio-Gel and Chelex 100 were obtained from Bio-Rad. All other materials were obtained from sources described previously (6).

Buffers—The following buffers were routinely used in these experiments: buffer A, 45 mM Hepes, 100 mM KCl, 5.7 mM MgCl2, 1 mM EGTA, (ethylenebis(oxyethylenetri)tetraacetic acid; SDS, sodium dodecyl sulfate.

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, (ethylenebis(oxyethylenetetrato)tetraacetic acid; SDS, sodium dodecyl sulfate.

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The method described by Reimann et al. (13) was used with a running buffer of pH 8.3.

Phosphorylase kinase (15), inhibitor 1 (16), and inhibitor 2 (17) were purified from rabbit skeletal muscle. An initial sample of inhibitor 2 was kindly donated by Dr. David Brautigan (Brown University).

Phosphatase activity was measured by incorporating the proteins with 250-500 µCi of [γ-32P]ATP (25-50 µM) into the reaction mixture (200 µl). The supernatant was then added to 5 ml of scintillation liquid. The reaction was stopped by acid precipitation as previously described.

Preparation of Cytosol—For the survey of the phosphatase population in erythrocyte cytosol, 200 ml of blood was drawn from human male adults into a Na3-heparin anticoagulant. All subsequent steps were carried out at 4°C in the presence of 100 µM phenylmethylsulfonyl fluoride, 250 µM mercaptoethanol, 10 µM sodium azide, pH 8.0. Prior to phosphorylation the solution was dialyzed against a buffer containing 20 mM Tris, 50 mM KCl, 1 mM MgCl2, pH 7.4. SDS-gel electrophoresis was routinely used to ensure that there was no breakdown of the two spectrin subunits into smaller fragments.

Phosphorylase kinase (5-10 mg/ml), spectrin (5-10 mg/ml), and casein (about 10 mg/ml) were phosphorylated by incubating the proteins with 250-500 µCi of [γ-32P]ATP (25-50 µM) in buffer A for 3-5 h at room temperature. Excess label was removed by precipitating casein with 10% trichloroacetic acid; 750 µl of the supernatant was then added to 5 ml of ScintiVerse and stored at 4°C in 0.3 mM phosphate, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM MgCl2, pH 7.4. SDS-gel electrophoresis was routinely used to ensure that there was no breakdown of the two spectrin subunits into smaller fragments.

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**Erythrocyte Phosphoprotein Phosphatase**

**Substrates—Erythrocyte pyruvate kinase was prepared as described previously (2). Casein was dephosphorylated by the method of Laemmli (22). Discontinuous acrylamide gels (5-15% gradient) were run and calibrated with standards: phosphorylase a or [32P]phosphocasein (100,000 cpm/10 µl); incubations were carried out at 4°C in the presence of 100 µM phenylmethylsulfonyl fluoride, 250 µM mercaptoethanol, 10 µM sodium azide, pH 8.0. Prior to phosphorylation the solution was dialyzed against a buffer containing 20 mM Tris, 50 mM KCl, 1 mM MgCl2, pH 7.4. SDS-gel electrophoresis was routinely used to ensure that there was no breakdown of the two spectrin subunits into smaller fragments.

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mm Tris, 50 mM KCl, 20 mM mercaptoethanol, 5% glycerol, 850 μM 
MnCl₂, pH 7.4, at 4 °C, at 150 V for 10 h (until no activity could 
be detected in the compartment containing the sliced gel). The enzyme 
solution was made 50% saturated in ammonium sulfate by adding the 
solid salt.

Gel Filtration—The ammonium sulfate suspension of protein from the 
non-denaturating gel was centrifuged and dissolved in 0.5 ml of 
buffer D. This solution was applied to an Ultrogel AcA 34 column 
(1.5 x 96 cm) and the protein eluted with buffer D; 1.2 ml samples 
were collected. Fractions possessing phosphatase activity of greater 
than 50% of the peak activity were pooled and made 50% saturated 
in ammonium sulfate.

Freezing and Thawing of the Phosphatase—The ammonium sulfate 
suspension of the phosphatase from the gel filtration column was 
centrifuged and the pellet redissolved at a concentration of about 1 
mg/ml protein in buffer B containing 15 mM mercaptoethanol. The 
solution was frozen on dry ice for 5 min and the sample, still frozen, 
centrifuged at 12,000 x g for 1 min at room temperature (Eppendorf 
Microfuge); during this time the samples melted. The supernatant 
was removed and assayed for activity and protein concentration and 
the freeze-thaw cycle repeated.

Octyl-Sepharose Chromatography—The 50% ammonium sulfate 
suspension of the phosphoprotein phosphatase from the gel filtration 
column was diluted with buffer C to a final concentration of 1 mM 
ammonium sulfate. The solution was passed through an octyl-Sepharose 
column (1 x 12 cm) previously equilibrated with buffer C 
containing 1 mM ammonium sulfate, pH 7.4, at 4 °C. The column was 
eluted with an ammonium sulfate gradient (2 x 500 ml; 1-0 M) in 
5 mM Hepes, 1 mM Mn²⁺, pH 7.4; 3-ml fractions were collected and 
assayed for phosphatase activity.

Sedimentation Velocity Measurements—These measurements were 
done in linear glycerol gradients with a density range of 1.01-1.07 
which had the salt and buffer compositions of buffer A. Mixtures of 
phosphatase with standards were layered on the gradients which were 
than centrifuged from between 14 and 16 h at 40,000 or 50,000 rpm 
in Beckman SW56 or SW60.1 rotors at 4 °C. Gradients were tapped 
by upward displacement and the 20-26 fractions were analyzed by 
enzyme activity and by absorbance at 220 nm for hemoglobin. Con-
siderations cited by Martin and Ames (24) were taken into account. 
As internal standards we used hemoglobin, lactate dehydrogenase, 
yeast alcohol dehydrogenase, and catalase with s₂₀,₅₀ values of 4.3, 7.3, 
10.6, and 11.3, respectively. Three different preparations of phospha-
tase were examined and on the last occasion an aliquot of the same 
phosphatase was simultaneously chromatographed on an Ultrogel 
AcA 34 column to establish that its gel permeation behavior was 
extactly as expected from previous measurements which had given a 
molecular weight relative to standards of 180,000.

RESULTS

Metal Activation Profile of Cytosolic Phosphatase—A survey of 
the different populations of phosphatase found in the human red blood cell 
cytosol is shown in Fig. 1; the forms can be distinguished by apparent molecular weight and differences 
in metal activation. The broken curve represents the activation of 
the different molecular weight forms by Mn²⁺ and the 
solid curve represents Mg²⁺-stimulated activity. The major 
Mn²⁺-activated peak is found at an equivalent sphere position 
of 180,000 Da and constitutes 60% of all phosphatase activity: 
in the presence of Mg²⁺ this enzyme constitutes only 20% of 
the total activity. Overall Mn²⁺ activation is seen to be more 
than 30-fold greater than Mg²⁺ activation. The phosphatase 
we discuss in this paper is the form labeled 180,000 Da in Fig. 
1.

Characterization of the Purified Holoenzyme and Its Cata-
lytic Subunit—The purpose of the adopted procedures was to 
purify the major phosphoprotein phosphatase activity without 
changing the molecular weight of the enzyme. For conven-
ience, enzymatic activity was routinely followed by assaying 
Mn²⁺-dependent hydrolysis of p-nitrophenyl phosphate but 

at all of the states of phosphospectrin, phosphocasein, and phosphopyruvate kinase phos-
patase activities were coincident with Mn²⁺-dependent p-
nitrophenylphosphatase activity. Activity against p-nitro-
phenyl phosphate and phosphocasein was proportional to 
the amount of enzyme added over a 10-fold range of concen-
tration, and the ratio of p-nitrophenylphosphatase activity to 
spectrin phosphatase activity remained constant. Activity 
against casein and phosphorylase was less simple; at higher 
concentrations of enzyme, enzymatic activity was not directly 
proportional to enzyme concentration but showed a relative 
increase in activity as the protein was diluted.

The overall purification is given in Table I. A purification 
of about 300,000-fold with a yield of up to 53% can be achieved 
routinely. Often during the preparation procedure there was 
a significant increase in overall phosphatase activity com-
pared with that seen in the 20-45% ammonium sulfate frac-
tion. This did not consistently occur at a particular point of 
the purification procedure and there was no apparent change 
in the subunit structure of the enzyme.

Molecular Weight—Gel filtration of the 25-45% ammonium 
sulfate fraction of the hemolysate and the purified enzyme 
showed one major peak corresponding to a relative molecular 
weight of 180,000 based on simple comparison to standard 
proteins (Fig. 2, A and B). Sedimentation rates in a glycerol 
gradient, however, indicated a much lower molecular weight. 
Four samples of three preparations of the phosphatase showed 
that the phosphatase sedimented behind lactic dehydrogenase 
(Mₚ = 140,000). Based on interpolation on a curve of sedi-
mentation distance versus s₂₀,₅₀ for the standard proteins, the 
s₂₀,₅₀ for the phosphatase is 6.1 ± 0.2. If we calculate s₂₀,₅₀ from
Erythrocyte Phosphoprotein Phosphatase 2019

TABLE I

| Sample                  | Enzymatic activity units $\times 10^{-3}$ | Protein mg | Specific activity nmol/min/mg | Purification | Yield |
|-------------------------|-------------------------------------------|------------|-------------------------------|--------------|-------|
| Hemolysate              | 17.6                                      | 132 $\times 10^3$ | 0.134                         | 1            | 100   |
| 20–45% (NH$_4$)$_2$SO$_4$ | 17.7                                      | 850        | 20.8                          | 150          | 100   |
| After dialysis           | 40.3                                      | 670        | 60.6                          | 452          | 228   |
| CM percolate             | 28.0                                      | 190        | 146                           | 1,090        | 158   |
| DEAE pool                | 38.3                                      | 20.0       | 1,907                         | 14,230       | 216   |
| Electroeluted enzyme     | 12.0                                      | 1.32       | 9,115                         | 68,020       | 64    |
| Gel filtration pool      | 9.4                                       | 0.21       | 44,600                        | 332,840      | 53    |

*Activity against p-nitrophenyl phosphate assay condition as described under "Materials and Methods."

![Fig. 2](image)

**Fig. 2.** Gel filtration of phosphoprotein phosphatase preparations on Ultrogel ACA 34 (1.6 $\times$ 100 cm). The samples were applied and eluted with buffer D at 4 °C. The column was calibrated with standards: ferritin, pyruvate kinase, $\gamma$-globulin, hemoglobin, and carbonic anhydrase; pyruvate kinase and hemoglobin were added to each phosphatase sample as internal standards. ☑, activity against p-nitrophenyl phosphate; ☐, activity against phosphocasein. A, 20–45% ammonium sulfate fraction of red blood cell lysate; B, purified enzyme; C, purified enzyme frozen and thawed three times; D, purified enzyme frozen and thawed seven times.

The gel filtration elution volume compared with the standard proteins and assume a $\eta$ of 0.725 we get a molecular weight estimate of 135,000, substantially lower than the 180,000 based on gel filtration alone. The elution of the phosphatase at the position of a higher molecular weight equivalent sphere suggests appreciable asymmetry in the holoenzyme. The Stokes radius of the phosphatase holoenzyme calculated from the gel filtration data is 58 Å.

If the holoenzyme was treated with acetone or ethanol, a procedure frequently used to generate active subunits of protein phosphatases, much activity was lost. This was true both after the ammonium sulfate fraction and after the pH 8 DEAE column (Fig. 3). After ethanol treatment of the ammonium sulfate fraction, the activity was lost.

![Fig. 3](image)

**Fig. 3.** Ion-exchange chromatography of the phosphoprotein phosphatase (prepared as described in the text). DEAE-A50 equilibrated with 20 mM Tris succinate, 1 mM MnCl$_2$, pH 8.0, eluted with 0.09–1 M KCl gradient (500 ml). ☑, p-nitrophenylphosphatase activity; ☐, spectrin phosphatase activity; ☐, absorbance at 280 nm; ☐, KCl concentrations.

Weight forms of the enzyme as detected by gel filtration (Fig. 2C). All show Mn$^{2+}$-simulated activity against both phosphocasein and p-nitrophenyl phosphate. After three or four cycles of freezing and thawing, the protein concentration of the sample dropped due to precipitation. Gel filtration of the supernatant still showed a major peak of activity at an equivalent sphere molecular weight of about 180,000 but a minor band of lower molecular weight was also visible (Fig. 2C). If these freeze-thaw cycles were continued more protein was removed; gel filtration of the enzyme after these freezing and thawing cycles showed that nearly all the activity eluted in a peak corresponding to an apparent molecular weight of about 36,000 (Fig. 2D). The transformation to the lower molecular weight form was greatly inhibited by the presence of 10% glycerol.

The effect of freezing and thawing on the enzyme suggested that hydrophobic interactions may be involved in the holoenzyme organization so the enzyme was chromatographed on octyl-Sepharose. Gel filtration of the enzyme eluted from the octyl-Sepharose column showed that the majority of the enzyme was converted to the 36.3 kDa form. This enzyme is identical (by gel filtration) to the lower molecular weight form of the frozen and thawed enzyme, which suggests that they are the same small subunit of the enzyme.

After several weeks, the pure holoenzyme, kept as an ammonium sulfate suspension in 10% glycerol at 4 °C, degrades into lower molecular weight forms. This may be due to pro-
teolysis from trace contaminants or to slow spontaneous alteration of the subunit structure. In the absence of glycerol, the enzyme degrades to the 36.3-kDa form over 24–36 h at 4 °C.

**Gel Electrophoresis**—From the densitometric scans of purified holoenzyme run on nondenaturing gel and stained with Coomassie Blue, it is possible to estimate that the active phosphatase band represents at least 90% of the protein from the Ultrogel column (Fig. 4A).

If the phosphatase preparation is either frozen and thawed or chromatographed on an octyl-Sepharose column, the active band of RF value 0.49 disappears and is replaced by one major active band of higher mobility, RF 0.68 (Fig. 4B).

Denaturing polyacrylamide electrophoresis of the purified enzyme shows two bands of equal intensity with molecular weights corresponding to 61,700 and 36,300 (Fig. 5A). If the two subunits stain equally with Coomassie Blue, then the

**Fig. 4.** Densitometric scans of purified phosphatase run on a nondenaturing gel (details in text) and stained with Coomassie Blue. A, holoenzyme; B, after freezing and thawing 7 times and centrifuging down insoluble material.

**Fig. 5.** Densitometric scans of purified phosphatase on a SDS denaturing gel (details in text) and stained with Coomassie Blue. A, holoenzyme; B, holoenzyme after freezing and thawing 7 times and centrifuging down any insoluble material. Standard positions shown at the top of the figures are: 1, phosphorylase (94 kDa); 2, bovine serum albumin (68 kDa); 3, ovalbumin (43 kDa); 4, carbonic anhydrase (29 kDa); 5, soybean trypsin inhibitor (21.5 kDa); 6, lysozyme (14.3 kDa).

The dissociated 62-kDa noncatalytic subunit precipitates out of solution under these conditions.

**Metal Dependence of Holoenzyme Activity**—Enzymatic activity of the holoenzyme against 32P04 from: A, pyruvate kinase; B, spectrin; C, casein (prepared as described in the text). A, with no phosphatase added; □, with phosphatase + 5 mM EDTA; ●, with phosphatase + 5.7 mM MgCl2; ○, with phosphatase + 1 mM MnCl2. Assays were performed as described under “Materials and Methods”; about 5 units of phosphatase was Chelex treated and then incubated in the appropriate buffer for 5 min prior to assay.

**Fig. 6.** Metal dependence of phosphoprotein phosphatase activity. Hydrolysis of 32P04, from: A, pyruvate kinase; B, spectrin; C, casein (prepared as described in the text). A, with no phosphatase added; □, with phosphatase + 5 mM EDTA; ●, with phosphatase + 5.7 mM MgCl2; ○, with phosphatase + 1 mM MnCl2. Assays were performed as described under “Materials and Methods”; about 5 units of phosphatase was Chelex treated and then incubated in the appropriate buffer for 5 min prior to assay.
Erythrocyte Phosphoprotein Phosphatase

80
60
40
20
0
200
400
300
CMn²⁺ (p M)

FIG. 7. Saturation curves for Mn²⁺ activation. A, activity against p-nitrophenyl phosphate. The line drawn is a theoretical one for a $K_a$ of 86 µM. B, activity against phosphocasein. The rate was measured as $^{32}$P0₄ released in 10 min at pH 7.4, 30 °C. In both graphs the residual activity which is present in the absence of metal has been subtracted from all points. The purified phosphatase was Chelex-treated prior to use; assays were done in buffer B with the indicated concentration of Mn²⁺ and 10 mM mercaptoethanol.

$K_{a5}$ ([Mn²⁺] at half of the apparent $V_{max}$) of about 24 µM was observed.

After treatment of the holoenzyme with 1 mM Mn²⁺ and subsequent removal of metal with EDTA, the kinetic properties of the enzyme, against all the substrates, are the same as those of enzyme purified without the addition of Mn²⁺. The effect of Mn²⁺ was found to be fully reversible. This is in contrast to the behavior reported on phosphatases from the rabbit muscle (13, 27). Although addition of Mn²⁺ to the lysate of freshly drawn blood does not cause a significant stimulation of activity, upon treatment of the lysate with Dowex 2, the phosphatase does become characteristically activated by Mn²⁺. Presumably Mn²⁺ activation prior to Dowex 2 treatment of the lysate is obscured by the high concentration of chelating ions found in the red blood cell cytosol. In the absence of Mn²⁺ the enzymatic activity is very sensitive to oxidized glutathione and air and is also labile to trypsin; in the presence of Mn²⁺ the enzyme is much more stable (data not shown).

The stimulation by Mg²⁺ does not approach saturation and it is not possible to obtain a value for $K_a$. Compared with Mn²⁺, the affinity of the enzyme for Mg²⁺ is much lower. Cobalt stimulates phosphatase activity to about the same extent as Mn²⁺, but this is only detectable after removal of protective sulfhydryl reagents. Calcium (0.5–3.5 mM) or cal-

cium and calmodulin (150 µg/ml) show no activation: millimolar concentrations of zinc are inhibitory, but micromolar concentrations show neither activation nor inhibition. Lack of inhibition by micromolar Zn²⁺ as well as sensitivity to fluoride (Table II) is characteristic of phosphoserine phosphatases in contrast to phosphotyrosyl phosphatase (28).

Metal Dependence of the Catalytic Subunit—As in the case of holoenzyme, the 36,300-Da catalytic subunit shows activity in the absence of added metal ion and in the presence of 5 mM EDTA, but is markedly stimulated by divalent cations (Fig. 8). The catalytic subunit differs from the holoenzyme in several respects. Upon conversion of the enzyme to the 36,300-Da subunit, by freezing and thawing, there is an increase in

| % hydrolysis | Casein | Pyruvate kinase | Spectrin |
|--------------|--------|-----------------|---------|
| No additions | 100    | 100             | 100     |
| 10 mM KF     | 78     | 60              | 76      |
| 10 mM ATP, 10 mM Mn²⁺ | 65     | 25              | 25      |
| 10 mM ADP, 10 mM Mn²⁺ | 54     | 25              | 25      |
| 10 mM Pi     | 22     |                 |         |
| 10 mM ATP    | 13     | 5               |         |
| 10 mM ADP    | 17     | 10              |         |
| 10 mM 2,3-bisphosphoglycerate | 35     | 46              |         |
| 10 mM pyrophosphate, 10 mM Mn²⁺ | 70     |                 |         |
| 5 mM EDTA    | 16     | 10              | 11      |

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|--------------|--------|-----------------|---------|
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| 10 mM KF     | 78     | 60              | 76      |
| 10 mM ATP, 10 mM Mn²⁺ | 65     | 25              | 25      |
| 10 mM ADP, 10 mM Mn²⁺ | 54     | 25              | 25      |
| 10 mM Pi     | 22     |                 |         |
| 10 mM ATP    | 13     | 5               |         |
| 10 mM ADP    | 17     | 10              |         |
| 10 mM 2,3-bisphosphoglycerate | 35     | 46              |         |
| 10 mM pyrophosphate, 10 mM Mn²⁺ | 70     |                 |         |
| 5 mM EDTA    | 16     | 10              | 11      |

The effect of Mn²⁺ was found to be fully reversible. This is in contrast to the behavior reported on phosphatases from the rabbit muscle (13, 27). Although addition of Mn²⁺ to the lysate of freshly drawn blood does not cause a significant stimulation of activity, upon treatment of the lysate with Dowex 2, the phosphatase does become characteristically activated by Mn²⁺. Presumably Mn²⁺ activation prior to Dowex 2 treatment of the lysate is obscured by the high concentration of chelating ions found in the red blood cell cytosol. In the absence of Mn²⁺ the enzymatic activity is very sensitive to oxidized glutathione and air and is also labile to trypsin; in the presence of Mn²⁺ the enzyme is much more stable (data not shown).

The stimulation by Mg²⁺ does not approach saturation and it is not possible to obtain a value for $K_a$. Compared with Mn²⁺, the affinity of the enzyme for Mg²⁺ is much lower. Cobalt stimulates phosphatase activity to about the same extent as Mn²⁺, but this is only detectable after removal of protective sulfhydryl reagents. Calcium (0.5–3.5 mM) or cal-

TABLE II

| % hydrolysis | Casein | Pyruvate kinase | Spectrin |
|--------------|--------|-----------------|---------|
| No additions | 100    | 100             | 100     |
| 10 mM KF     | 78     | 60              | 76      |
| 10 mM ATP, 10 mM Mn²⁺ | 65     | 25              | 25      |
| 10 mM ADP, 10 mM Mn²⁺ | 54     | 25              | 25      |
| 10 mM Pi     | 22     |                 |         |
| 10 mM ATP    | 13     | 5               |         |
| 10 mM ADP    | 17     | 10              |         |
| 10 mM 2,3-bisphosphoglycerate | 35     | 46              |         |
| 10 mM pyrophosphate, 10 mM Mn²⁺ | 70     |                 |         |
| 5 mM EDTA    | 16     | 10              | 11      |
total Mn$^{2+}$-stimulated activity but there is no marked change in the $K_m$ for Mn$^{2+}$. In this respect the larger subunit would appear inhibitory. The activity of the catalytic subunit is also more effectively stimulated by Mg$^{2+}$ than is the activity of the holoenzyme; activity approaches that achieved upon Mn$^{2+}$ stimulation (Fig. 8). This change may arise both from a change in the affinity of Mg$^{2+}$ for the enzyme and an increase in maximum velocity upon removal of the 61,700 noncatalytic subunit. Under normal assay conditions, in the presence of either 5.7 mM MgCl$_2$ or 1 mM MnCl$_2$, there is an 8-fold increase in Mg$^{2+}$-stimulated activity and a 2–3-fold increase in Mn$^{2+}$-stimulated activity when the holoenzyme is converted to the catalytic subunit.

Substrate Profile—The high molecular weight phosphatase shows activity against a broad range of substrates. Those hydrolyzed include: p-nitrophenyl phosphate, phosphospectrin, phosphorylase, phosphorylase kinase ($\beta$ subunit), the regulatory subunit of cAMP-dependent protein kinase, phosphocasein, and phosphorylated pyruvate kinase.

The enzyme shows very little activity towards simple phosphate esters other than p-nitrophenyl phosphate. Eleven low molecular weight phosphate esters tested were used as substrates at 30°C in buffer C at initial substrate concentrations of 1 mM. The phosphate release after 2 h incubation with 100 units of purified phosphatase was measured and compared to a control without phosphatase. The most active substrates were threonine phosphate and phosphoethanolamine; 13% of their phosphate was released. That is 0.8% of the rate of hydrolysis of p-nitrophenyl phosphate. Fruuctose 1,6-bisphosphate and serine phosphate were hydrolyzed at about half that rate. Phosphoethanolamine, phosphoglycolate, and glucose 6-phosphate were hydrolyzed at about 0.08% of the rate of p-nitrophenyl phosphate and 2,3-bisphosphoglycerate, 2-phosphoglycerate, ATP, and ADP were all hydrolyzed at half that rate or less. Because the degree of hydrolysis is low these rates should approximate initial rates of hydrolysis.

The substrate saturation curve for activity against phosphoproteins enzyme appears to follow simple Michaelis-Menten kinetics. However, it was not possible to obtain phosphoprotected spectrin in sufficient concentration to observe full saturation of the enzyme in this assay system. From data obtained using up to 11 $\mu$M protein-bound phosphate we estimated values of 10 $\mu$M (protein bound P) for $K_m$.

Phosphorylation of spectrin yielded labeled protein containing 1–2 mol of radioactive phosphate/mole of spectrin $\beta$ subunit compared with the reported maximum of 4 phosphates/$\beta$ subunit. The less than maximal incorporation of $^{32}$P may be due to the fact that the spectrin used was not dephosphorylated prior to phosphorylation. SDS-gel electrophoresis showed that at least 80% of the radioactive label could be attributed to spectrin labeled in the $\beta$ subunit. If we assume that the phosphatase does not distinguish among the phosphate groups on the $\beta$ subunit and assuming 4 mol of P$_i$/mol of $\beta$ subunit, then the $K_m$ based on spectrin $\beta$ subunits would be of the order of 6 $\mu$M.

In this laboratory pyruvate kinase has been phosphorylated to the level of 2–3 mol of P$_i$/mol of pyruvate kinase tetramer, with a reported maximum of 4 mol of P$_i$/mol of enzyme (6). D Dephosphorylation of pyruvate kinase shows simple saturation kinetics, similar to those found for spectrin dephosphorylation. Based on a maximum value of 1 mol of P$_i$/mol of pyruvate kinase subunit, a $K_m$ of 18 $\mu$M (protein-bound phosphate) or 24 $\mu$M (subunit pyruvate kinase), is obtained.

Dephosphorylation of casein by the holoenzyme also shows apparent Michaelis-Menten kinetics with an apparent $K_m$ of 63 $\mu$M casein. However, casein prepared as described by Reimann and co-workers (13) was found to still contain at least 95% of the original phosphate groups leaving a maximum of 5% of the sites available for phosphorylation with radioactive label. Thus dephosphorylation of the labeled phosphate groups may not be representative of overall dephosphorylation. Assuming that all phosphate groups of casein are hydrolyzed at similar rates, the maximum velocity for casein dephosphorylation showed a specific activity between 0.4 and 1.7 $\mu$mol/min/mg of phosphatase.

Subject to the uncertainties described above, we can summarize the maximum velocities of the 3 protein substrates in buffer C at 30°C. Spectrin, pyruvate kinase, and casein show maximum velocities within a factor of two of each other at about 1.0–2.0 $\mu$mol/min/mg of phosphatase. This is 2–4% of the rate obtained with $p$-nitrophenyl phosphate which is 44 $\mu$mol/min/mg enzyme or 44 X 10$^3$ units/mg of phosphatase. The low molecular weight phosphate esters are hydrolyzed at one-tenth the rate of the protein substances at similar concentrations.

The catalytic subunit shows the same substrate specificity as the holoenzyme.

Relative Specificity for Phosphorylase Kinase $\alpha$ and $\beta$ Units—Assays were carried out as described under "Materials and Methods." Densitometric scans of the autoradiogram of phosphorylase kinase showed there to be an approximate 1:1 labeling of the $\alpha$ and $\beta$ subunits (Fig. 9A). After 30 min the fraction incubated in the presence of the holoenzyme showed a 10% loss in label in the $\alpha$ subunit and a 79% loss in the $\beta$ subunit (Fig. 9B).

The catalytic subunit showed similar specificity. Under the same conditions it caused a 6% loss of label in the $\alpha$ subunit and a 72% loss in the $\beta$ subunit (Fig. 9C).

Inhibition of the Phosphatase—Since the enzyme is markedly sensitive to the presence of Mn$^{2+}$, any chelating agent is likely to have a pronounced effect on the enzymatic activity. EDTA, ADP, and ATP, all quite strongly inhibit activity against phosphocasein (Table II). In the presence of higher concentrations of Mn$^{2+}$, inhibition can still be observed but to lesser extents; ADP and ATP show the most inhibition. It was not possible to measure inhibition by 2,3-bisphosphoglycerate in the presence of higher Mn$^{2+}$ due to precipitation of the metal. Dephosphorylation of both pyruvate kinase and spectrin is markedly inhibited by ADP:Mn$^{2+}$ and ATP:Mn$^{2+}$; again KF inhibited but less strongly (Table II). The catalytic subunit showed an identical inhibition profile.

Effect of Rabbit Muscle Inhibitors 1 and 2 on Phosphatase Activity—Sensitivity to rabbit muscle inhibitors 1 and 2 is a distinguishing characteristic of type 1 phosphoprotein phosphatases. When measured with phosphorylase a as a substrate, both the holoenzyme and the catalytic subunit were inhibited 40–50% by the same concentrations of inhibitors 1 or 2 that inhibited rabbit muscle protein phosphatase 50% under equivalent circumstances. Similar degrees of inhibition were found with phosphocasein as substrate.

pH Profile and Isoelectric Point—The pH dependence of activity against phosphocasein and $p$-nitrophenyl phosphate shows a broad profile with an optimum around pH 7.4.

Isoelectric focusing of the purified enzyme proved to be quite difficult because often activity was lost during the focusing, or the protein precipitated out in the gel. However, preliminary data from wide pH range pre-pore gels, pH 3.5–9.5, indicated that the enzyme has a pI of about 5.0. This is in agreement with the characteristics of the enzyme shown during ion-exchange chromatography.
DISCUSSION

Although 30,000–35,000 catalytic subunits of phosphoprotein phosphatases have been purified from a number of tissues, purification of the higher molecular weight forms found in crude extracts has proved difficult. Iagebritsen and Cohen (26) have proposed a useful classification of protein phosphatases based on substrate specificity, metal activation, and sensitivity to protein inhibitors 1 and 2. Very recently high molecular weight forms of three type 2 phosphatases have been purified (22, 30, 31). These enzymes act preferentially on the α subunit of phosphorylase kinase and are not inhibited by protein inhibitors 1 and 2 (32). The enzyme we have purified is, by these same criteria, clearly a type 1 phosphatase. Purification of a type 1 phosphatase from liver glycogen particles has been reported by Straffors et al. (33). That enzyme appears to be a dimer of 1 catalytic subunit (37,000 Da) and one other subunit (103,000 Da) which causes binding of the enzyme to glycogen particles. The noncatalytic subunit is thus much larger than the one we find but it may have a similar function. In the enzyme we have isolated, the 62-kDa subunit has an inhibitory effect when activities are measured with Mg²⁺ as activator (presumably the physiological condition). It also appears to mediate binding of the enzyme to the erythrocyte membrane. We have shown that the enzyme binds reversibly to the membrane and that the membrane-bound form is inactive (34, 35). Inside-out red cell membrane vesicles will selectively remove only the holoenzyme form of the enzyme from crude preparations (37) but experiments on binding of purified holoenzyme and catalytic subunit to red cell membranes have given equivocal results (25, 38).

Gruppuso et al. (36) have shown that in muscle extracts there is a 60-kDa protein that cross-reacts immunologically with inhibitor 2 and that in a crude fraction of phosphatase, tryptic digestion leads to phosphatase activation in step with destruction of the 60-kDa antigen. Samples of our holoenzyme have been examined by Western blotting in Dr. Brautigan’s laboratory but no evidence of cross-reaction with inhibitor 2 antibody was found.

A high molecular weight protein phosphatase purified from human erythrocytes by Usui et al. (8) is particularly interesting for its relationship with the enzyme we have purified. The 104,000-Da enzyme purified by Usui et al. is the major Mg²⁺-activated enzyme of the red cell and is composed of one 32,000-Da catalytic subunit and one 69,000-Da subunit. It was not tested for activity toward phosphorylase kinase α and β subunits but was judged to be a type 2 enzyme because it was not inhibited by rabbit muscle inhibitor 2. That enzyme was also completely inhibited by Mg²⁺ concentration that fully activates the enzyme we purified. The enzyme that we purified is the enzyme designated phosphatase I in the survey by Usui et al.; the one they purified was their phosphatase IV. Using spectrin phosphate activity as the point of comparison of the two papers, we find that the papers are in agreement on the specific activity of the homogenate and on the relative amounts of the enzyme forms.

Two points need mention: Usui et al. (8) reported the same Stokes radius for the crude “phosphatase I” that we report for the purified enzyme, but they found an s₂₀,ₐ₀ for that enzyme of 7.4 while we find 6.1 ± 0.2. They therefore calculate a molecular weight of 180,000 for the crude enzyme in agreement with the gel permeation estimate. Our sedimentation results show a much more asymmetric molecule with a molecular weight of about 135,000. A second point of apparent disagreement is that we find that our enzyme is sensitive to inhibitors 1 and 2 of rabbit muscle, using inhibitors prepared in this laboratory and also a sample of inhibitor 2 generously provided by Dr. David Brautigan. Rabbit muscle protein phosphatase was used as a positive control and to assess the activity of the inhibitor preparations. Usui et al. reported that none of the erythrocyte phosphatases were sensitive to inhibitor 2 but did not report a positive control. The possibility exists that the purified enzyme which we studied shows properties different from the enzyme in the crude homogenate, but since substrate specificity, gel permeation behavior, and metal ion specificity do not change during purification that possibility is not high.

The size of the catalytic subunit and the noncatalytic subunits in the enzyme we have purified are very similar to those of the type 2 enzymes isolated from rabbit skeletal muscle (29), rabbit heart (30), and turkey gizzard (31). In none of these cases has the function of the large subunit been understood and despite the type 1-type 2 differences, it may be that these enzymes are much closer in structure and function than is apparent now.

The meaning of Mn²⁺ activation is also a problem for future work. Our current expectation is that Mn²⁺ elicits an activity which is cryptic under physiological conditions and that a physiological activator is to be discovered.
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