Purification and Characterization of Human Erythrocyte Purine Nucleoside Phosphorylase and Its Subunits*

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Purine nucleoside phosphorylase (EC 2.4.2.1; purine nucleoside:orthophosphate ribosyltransferase) from fresh human erythrocytes has been purified to homogeneity in two steps with an overall yield of 56%. The purification involves DEAE-Sephadex chromatography followed by affinity chromatography on a column of Sepharose/formycin B. This scheme is suitable for purification of the phosphorylase from as little as 0.1 ml of packed erythrocytes. The native enzyme appears to be a trimer with native molecular weight of 93,800 and the subunit molecular weight of 29,700 ± 1,100. Two-dimensional gel electrophoresis of the purified enzyme under denaturing conditions revealed four major separable subunits (numbered 1 to 4) with the same molecular weight. The apparent isoelectric points of subunits 1 to 4 in 9.5 M urea are 6.83, 6.41, 6.29, and 6.20, respectively. The different subunits are likely the result of post-translational modification of the enzyme and provide an explanation of the complex native isoelectric focusing pattern of purine nucleoside phosphorylase from erythrocytes. Three of the four subunits are detectable in two-dimensional electrophoretic gels of crude hemolysates. Knowing the location of the subunits of purine nucleoside phosphorylase in a two-dimensional electropherogram allows one to characterize the purine nucleoside phosphorylase in crude cell extracts from individuals with variant or mutant purine nucleoside phosphorylase as demonstrated in a subsequent communication. Partial purification of the phosphorylase from 1 ml of erythrocytes on DEAE-Sephadex increases the sensitivity of the detection of the subunits to the 0.3% level.

Purine nucleoside phosphorylase from mammalian sources reversibly catalyzes the phosphorylization of naturally occurring purine ribo- or 2'-deoxyribonucleosides with the possible exception of adenosine (1, 5). Several purine analogs such as 8-azaguanine (6), 6-mercaptopurine (7, 8), allopurinol, and oxipurinol (9) and their corresponding nucleosides can also serve as substrates. The equilibrium of the reaction catalyzed by the phosphorylase favors nucleoside synthesis (10, 11). The enzyme has also been reported to catalyze ribosyl transfer reactions in the absence of inorganic phosphate (12, 18), but this additional activity remains uncertain (11, 14, 15). The literature pertaining to this enzyme has been reviewed by Friedkin and Kalckar (10) and by Parks and Agarwal (11). The enzymes have been purified from human erythrocytes (13, 14), bovine spleen (16), bovine brain (17), and rabbit and Chinese hamster liver (18, 19). Chemical and genetic evidence suggest that the human enzyme is a trimer with a subunit molecular weight of 30,000 (13, 20, 21). However, differences both in the subunit structure and molecular weight have been reported for the mammalian enzymes from different sources (17, 18). Although genetic evidence suggests that in humans there is a single gene locus for purine nucleoside phosphorylase, the enzyme exhibits a variable degree of heterogeneity, depending on the tissue of origin (21). Starch gel electrophoresis has revealed one or two isozymes from fibroblasts and at least seven isozymes from erythrocytes, while enzymes from other tissues have intermediate numbers of isozymes (21).

Several rare variant alleles of purine nucleoside phosphorylase in the heterozygous state have been observed in normal subjects (21-23) and several patients with homozygous deficiencies of purine nucleoside phosphorylase have been reported (22, 24, 25). The patients with enzyme deficiencies have associated abnormal thymus-dependent lymphocyte function in the presence of normal humoral immunity. This suggests a role for purine nucleoside phosphorylase in the immune system.

The purpose of this study is to investigate further the nature of the heterogeneity and other properties of purine nucleoside phosphorylase in normal persons and to develop procedures for the purification and characterization of small quantities of the purine nucleoside phosphorylase subunit protein in normal, variant, and deficient human subjects. Except in abstract form (20), there are no published data convincingly describing the molecular weight of the subunits from demonstrably homogeneous human purine nucleoside phosphorylase. Therefore, it was necessary to purify the normal human erythrocytic enzyme to homogeneity, to determine unequivocally the subunit molecular weight and to verify the molecular weight of the native enzyme (13, 21).

By means of a denaturing two-dimensional electrophoretic

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EXPERIMENTAL PROCEDURES

Materials—Inosine, hypoxanthine, formin B, and bovine serum albumin were purchased from Calbiochem, and ultrapure grade urea was purchased from Schwarz/Mann. Tris (2-carboxyethyl)phosphate buffer, pH 7.4, 1 mM inosine, and 0.025 unit of xanthine oxidase/ml. The absorbance at 293 nm due to the formation of uric acid was monitored on a recording Zeiss spectrophotometer. Protein concentrations were estimated by the method of Lowry et al. (27) with bovine serum albumin as the standard.

Preparation of Sepharose 4B/Formycin B Affinity Column—Commercially available activated Sepharose 4B and material prepared according to Cuatrecasas and Anfinsen (28) were used. The activated beads were washed briefly with a solution of 2 M 3,3′-iminobispropylenediamine adjusted to pH 10 and then were suspended in equal volume of Buffer 1.

Enzyme Purification—The purification was carried out at 4°C. Enzyme preparations were kept in equal volume of Buffer 1 stored in equal volume of Buffer 1.

Linking of the derivatized Sepharose 4B to formin B was carried out by the method of Wichek and Lamed with minor modifications (29). A 10-ml solution of 50 mM formycin B was placed in a dark plastic bottle, adjusted to pH 7 to 8, and oxidized by the addition of solid sodium periodate to a final concentration of 47.5 mM. The reaction mixture was allowed to react at 4°C in the dark for 1 h with constant rotation. A 10-ml aliquot of packed iminobispropyline-Sepharose 4B in Buffer 1 was added to the oxidized formin B solution and allowed to react at 4°C for 3 h. Three or four sequential additions of sodium borohydride (10 mg/ml of Sepharose) were then introduced into the reaction mixture at intervals of 1 h. The resulting derivatized Sepharose was filtered, washed with 1 liter of water, and introduced into a 12-ml disposable plastic syringe.

This affinity column was washed sequentially with 100 ml of Buffer a, 50 ml of Buffer a containing 1 mg of bovine serum albumin/ml, 100 ml of Buffer h, and finally with 150 ml of Buffer a. Similar treatment was used in order to reuse the affinity column.

Enzyme Purification—The purification was carried out at 4°C throughout. The washed erythrocytes from 130 ml of freshly drawn blood were suspended in 85 ml of Buffer a, lysed by freezing and thawing twice, and sonicated for 10 s.

The crude hemolysate was mixed with the minimal amount of DEAE-Sephadex (A-50) in Buffer a (Table I) sufficient to absorb 98% of the total purine nucleoside phosphorylase activity (approximately 100 ml). The suspension was stirred for 15 min and filtered and washed on a Buchner funnel with 0.5 liter of Buffer b. The beads were then removed from the funnel, stirred with 200 ml of Buffer c, filtered and washed as above; the washing procedure was then repeated with Buffer d. Finally the Sephadex A-50 beads were suspended in 100 ml of Buffer d, poured into a column (75 x 2.5 cm) and washed overnight with Buffer d. This material contained 20% of the starting activity and is referred to as "Affinity Fraction c" under "Results" and in Fig. 4C. The column was then stepwise eluted with Buffer e, and the fractions containing 55% of the starting activity were pooled for use for affinity chromatography, the next step of the purification. An additional 4 to 5% of the loaded purine nucleoside phosphorylase activity was eluted with Buffer e and subsequently with Buffer g. This latter material is referred to as DEAE-Fraction D under "Results" and in Fig. 4D. A total of 92% of the loaded purine nucleoside phosphorylase activity could be recovered from the DEAE-Sephadex column.

Eighty-four units of purine nucleoside phosphorylase from the affinity fraction were loaded onto a 12-cm column of hydroxyapatite equilibrated with Buffer j. The column was washed with 200 ml of Buffer j, and the purified nucleoside phosphorylase activity was eluted with Buffer k.

| Table I |

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Composition referred to in this report</th>
<th>pH</th>
<th>Conductivity at 25°C (mho/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>20 mM Tris/Cl, 6 mM MgCl₂, 0.2 mM DTT*</td>
<td>7.8</td>
<td>1.8</td>
</tr>
<tr>
<td>b</td>
<td>Buffer a plus 20 mM KCl</td>
<td>7.8</td>
<td>3.1</td>
</tr>
<tr>
<td>c</td>
<td>Buffer a plus 40 mM KCl</td>
<td>7.8</td>
<td>4.1</td>
</tr>
<tr>
<td>d</td>
<td>Buffer a plus 50 mM KCl</td>
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<td>5.0</td>
</tr>
<tr>
<td>e</td>
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<td>7.8</td>
<td>6.3</td>
</tr>
<tr>
<td>f</td>
<td>Buffer a plus 100 mM KCl</td>
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<td>8.0</td>
</tr>
<tr>
<td>g</td>
<td>Buffer a plus 150 mM KCl</td>
<td>7.8</td>
<td>11.0</td>
</tr>
<tr>
<td>h</td>
<td>Buffer a plus 1.5 M KCl</td>
<td>7.8</td>
<td>70</td>
</tr>
<tr>
<td>i</td>
<td>Buffer a plus 140 mM NaCl</td>
<td>7.8</td>
<td>10.6</td>
</tr>
<tr>
<td>j</td>
<td>48 mM potassium phosphate, 0.2 mM DTT</td>
<td>7.4</td>
<td>6.0</td>
</tr>
<tr>
<td>k</td>
<td>74 mM potassium phosphate, 0.2 mM DTT</td>
<td>7.4</td>
<td>7.3</td>
</tr>
<tr>
<td>l</td>
<td>200 mM Tricine, 1 M NaCl</td>
<td>8.2</td>
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</tbody>
</table>

* DTT: diithothreitol

Tricine, N-tris(hydroxymethyl)methylglycine.

| Table II |

Purification of purine nucleoside phosphorylase from normal human erythrocytes

The purification procedures are described under "Experimental Procedures." One unit of enzyme activity equals 1 pmol of product formed/min at 25°C. Part of the material from each step was applied to the subsequent purification procedure, as indicated in the table.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Loaded</th>
<th>Recovered</th>
<th>Overall yield</th>
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<tr>
<td></td>
<td>units/ml</td>
<td>mg/ml</td>
<td>units/mg protein</td>
<td>-fold</td>
<td>units</td>
<td>units</td>
<td>%</td>
</tr>
<tr>
<td>Crude hemolysate</td>
<td>3.04</td>
<td>154</td>
<td>0.0198</td>
<td>1.0</td>
<td>456</td>
<td>456</td>
<td>66.6</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 chromatography</td>
<td>0.27</td>
<td>0.12</td>
<td>2.43</td>
<td>123</td>
<td>253</td>
<td>253</td>
<td>66.6</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>1.79</td>
<td>0.031</td>
<td>55.0</td>
<td>2778</td>
<td>116</td>
<td>117.2</td>
<td>55.9</td>
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<tr>
<td>Hydroxylapatite chromatography</td>
<td>0.64</td>
<td>0.012</td>
<td>53.3</td>
<td>2692</td>
<td>84.3</td>
<td>65.5</td>
<td>43.4</td>
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</tbody>
</table>
Single-step Partial Purification of Phosphorylase - Single-step partial purification of erythrocyte purine nucleoside phosphorylase by ion exchange chromatography utilized 3 ml of DEAE-Sephadex A-50/ml of packed erythrocytes. A crude hemolysate was absorbed on a column of the Sephadex A-50, previously equilibrated with Buffer a. The column was then washed with 20 volumes of Buffer c and eluted with Buffer f. This step provided 100-fold purification, a yield of 70 to 80%, and an increase in the concentration of the alkali subunits.

Single-step partial purification by affinity chromatography utilized 1 ml of Sepharose/formycin B per ml of packed erythrocytes. The affinity column was eluted as described above. This step provided at least 1000-fold purification and a yield of 30 to 35%. The fractions containing the purine nucleoside phosphorylase activity were pooled, dialyzed, and lyophilized to be analyzed by the two-dimensional gel electrophoreses.

Slab Gel Electrophoresis - Electrophoresis under non-denaturing conditions was performed at 4°C on a vertical slab gel apparatus purchased from Hoefer or constructed according to the design described by Studier (30). The separating gels contained 4.1, 6.1, 8.2, 10.2, or 12.3% acrylamide and were prepared by the methods of Davis (31). The estimation of the native molecular weight of purified nucleoside phosphorylase by the method of Hedrick and Smith (32) is based on the electrophoretic mobility of the enzyme as a function of acrylamide concentration. Myoglobin, ovalbumin, and the monomers and dimers of bovine serum albumin were used as molecular weight markers.

The denaturing electrophoreses were in 12.3% acrylamide containing 0.1% sodium dodecyl sulfate. The protein markers and their subunit molecular weights were: bovine serum albumin, 66,000; ovalbumin, 43,000; aldolase, 40,000; chymotrypsinogen, 25,700; myoglobin, 17,200; and ribonuclease, 13,700.

Sample Preparations for Two-dimensional Electrophoretic Analyses - The erythrocytes which were used for the two-dimensional gel electrophoreses of crude hemolysates were washed extensively by centrifugation with phosphate-buffered saline to remove plasma proteins and were hemolyzed by suspending them in 2 volumes of a solution to give final concentrations of 8.8 M urea, 0.7 M β-mercaptoethanol, 1.6% amorpholines, pH 5 to 8, 0.4% amorpholines, pH 3.5 to 10.

Other materials analyzed by the two-dimensional system were dialyzed extensively against 14 mM β-mercaptoethanol at 4°C, lyophilized to dryness, and if not analyzed promptly, stored at -70°C until analysis.

Fig. 1. Polyacrylamide-sodium dodecyl sulfate gel electrophoresis of human erythrocyte purine nucleoside phosphorylase. The slab gel electrophoresis was carried out as described under “Experimental Procedures.” The samples are: A, 75 μg of DEAE-pool fraction eluted with 90 mM KCl and subsequently used for affinity chromatography; B, 6 μg each of molecular weight standards, from top to bottom: bovine serum albumin, ovalbumin, aldolase, chymotrypsinogen, and ribonuclease; C, 27 μg of the phosphorylase obtained by hydroxylapatite chromatography following the affinity chromatography; D, 31 μg of the affinity chromatography fraction of purine nucleoside phosphorylase.

Fig. 2. Coincidence of protein and the catalytic activity of purified human erythrocyte purine nucleoside phosphorylase detected by histochemical stain. Tracks A, B, and C represent strips of 6.1, 8.2, and 12.3% native acrylamide gels stained for proteins with Coomassie brilliant blue. The tracks A’, B’, and C’ show the histochemically stained purine nucleoside phosphorylase on the identical strips. Each track contained 14 μg of purified purine nucleoside phosphorylase.

Fig. 3. Native isoelectric focusing of human erythrocyte purine nucleoside phosphorylase on a slab of polyacrylamide. Isoelectric focusing and histochemical staining of purine nucleoside phosphorylase was performed as described under “Experimental Procedures.” The bands represent enzyme activity detected by the histochemical stain. Track A contained 0.18 unit of crude hemolysate; B, 1.9 units of purified purine nucleoside phosphorylase; C, 1.9 units of purified purine nucleoside phosphorylase previously treated with 1.6 μM 5,5′-dithiobis(2-nitrobenzoic acid) as described under “Experimental Procedures;” D, 0.95 unit of purified purine nucleoside phosphorylase treated with 4.5 M urea, 0.35 M mercaptoethanol for 10 min prior to applying to the gels. The gradient of pH at the completion of isoelectric focusing is indicated.
Two-dimensional Gel Electrophoresis — The apparatus, procedures, and buffers similar to those described by O’Farrell (33) were used with the following modifications. The pH 5 to 7 ampholines were replaced by pH 5 to 8 ampholines, the samples were loaded on the first dimension without prerunning, and the equilibration time between the two dimensions was limited to 15 min. The gels were stained in 2.5% Coomassie brilliant blue, 50% methanol, 7.5% acetic acid and destained in 7.5% acetic acid. The pH gradient in the first dimension was determined by extracting 0.5-mm slices of the isoelectric focused disc gel in water for several hours and measuring the pH of the solution with a combination electrode.

Native Acrylamide Isoelectric Focusing — The isoelectric focusing contained 5% acrylamide, 0.293% bisacrylamide, 0.96% total ampholines (pH 3.5 to 10, 0.4%; pH 4 to 6, 0.24%; and pH 5 to 8, 0.32%), ammonium persulfate, 0.68%, N,N,N′,N′-tetramethylethylenediamine, 0.057% by volume. Slabs of polyacrylamide gel (0.6 mm thick, 23 × 15 cm) were poured into a chamber, one face of which was glass and the other lucite. After polymerization, the spacers were removed and the acrylamide slab which remained attached to the glass plate was inverted and placed on top of two graphite electrodes which had been covered with 1.5-cm strips of Whatman paper, one (anode) saturated with 1% phosphoric acid and the other (cathode) with 2% ethanolamine. The gel was prefocused for 2 h at 400 V. The samples were applied directly to the gel surface in volumes up to 40 μl along linear tracks, the ends of which were oriented toward the electrodes. The gel was then focused at 250 V overnight, 400 V for 2 h, and 800 V for 2 h. The focusing was terminated when the hemoglobin marker bands which had been loaded on two adjacent channels on opposite ends of the gel were superimposed. The pH gradient was determined by extracting 1-cm gel slices for several hours in water and measuring the pH of the resulting solution by a combination electrode.

The purine nucleoside phosphorylase catalytic activity was located on the acrylamide gel slab by means of a specific histochemical stain containing phenazine methosulfate, 0.06 mg/ml; nitro blue tetrazolium, 0.6 mg/ml; xanthine oxidase, 0.063 unit/ml; 1 mM inosine, and 50 mM potassium phosphate buffer, pH 7.4. The isoelectric focusing gel was equilibrated for 30 min in 50 mM potassium phosphate buffer, pH 7.4, with three changes in order to prevent the basic end of the gel from catalyzing the reduction of phenazine.

**Fig. 4.** Two-dimensional gel electrophoresis of human erythrocyte purine nucleoside phosphorylase under denaturing conditions. The two-dimensional analyses were performed as described under “Experimental Procedures” and in Ref. 33. A, 39 μg of the affinity chromatographic fraction of purine nucleoside phosphorylase. The numbering of the subunits and the pH gradient are indicated. SDS, sodium dodecyl sulfate; IEF, isoelectric fractionation. B to F represent magnified portions of the two-dimensional gels in the vicinity of purine nucleoside phosphorylase. The visible subunits are numbered, and two marker proteins visible in B, C, and D are indicated (○). B, 75 μg of 120-fold purified DEAE-pool fraction eluted with buffer containing 90 mM KCl and subsequently used for affinity chromatography. C, 125 μg of DEAE-fraction C which was eluted with buffer containing 50 mM KCl. D, 600 μg of DEAE-fraction D which was eluted with buffer containing 150 mM KCl, as described under “Experimental Procedures.” (Note that the order of elution of these fractions is C, B, D).
RESULTS

Purification—Table II summarizes the purification of purine nucleoside phosphorylase. A two-step purification process resulted in at least 99.5% homogeneous enzyme with a specific catalytic activity of 55 units (μ mole/min) per mg of protein and a yield of 56%. A total of 92% of the loaded catalytic activity could be recovered from the DEAE-Sephadex column. However, only those fractions with high specific activity, representing 55% of the starting catalytic activity, were pooled and used for further purification and other experiments.

The affinity chromatography yielded 100% of the loaded activity and an enzyme with a specific catalytic activity of 55 units/mg of protein. Subsequent chromatography on hydroxyapatite did not afford any significant further purification. However, if the hydroxyapatite step is applied after the DEAE-Sephadex A-50 chromatography, an enzyme fraction with a specific activity of 20 units/mg can be obtained.

Fig. 1 shows the analysis of the various enzyme fractions by electrophoresis on sodium dodecyl sulfate-acrylamide slab gels. Even when a 37-μg aliquot of the affinity fraction was loaded on the gel (track D), impurities could barely be detected and were estimated to be less than 0.5% by densitometric scanning of the electropherogram. Even fewer impurities could be seen in the material chromatographed on hydroxyapatite (track C) following affinity chromatography.

Enzyme Stability—The purified purine nucleoside phosphor-ylase is stable in solution when stored at 4° in the presence of 0.025% NaN₃ and Buffer i. For example, an enzyme solution of 100 μg/ml retained 90% of the activity over a 3-month period at 4°. Storage of the same enzyme solution at −20° resulted in 50% loss of activity over 2 weeks. The addition of 1 mg of bovine serum albumin/ml increases further the stability of the enzyme upon storage at 4°.

Molecular Weights—The subunit molecular weight of human erythrocytic purine nucleoside phosphorylase, obtained from an analysis of 12 independent sodium dodecyl sulfate-acrylamide gel electrophoreses, is 29,700 ± 1,100. A native molecular weight of 93,800 was estimated from the electrophoretic mobility of the purified enzyme in various acrylamide concentrations by the method of Hedrick and Smith (32). As can be seen in Fig. 2, the purine nucleoside phosphorylase catalytic activity and the protein stain coincide at three different gel concentrations.

Native Isoelectric Focusing—Isoelectric focusing under native conditions on slabs of acrylamide gels as depicted in Fig. 3 reveals a broad area of enzymatic activity between pH 5.0 and pH 6.1. Individual isozymes are poorly distinguished by the histochemical staining method. The purified affinity fraction (track B) provides the same isozyme pattern as the crude hemolysate (track A), and the isozyme pattern did not change when the purified enzyme was treated with 4.5 M urea and 350 mM β-mercaptoethanol (track D) for 10 min prior to applying to the native gel. When the gel was subsequently stained with Coomassie brilliant blue, the stained protein in track B coincided with the catalytic activity of purine nucleoside phosphorylase (not shown). Reaction of purine nucleoside phosphorylase with 5,5'-dithiobis(2-nitrobenzoic acid) leading
to a 70% inactivation caused small changes in the isozyme distributions; some of the basic isozymes were lost while the acidic isozyme bands became more distinct (track C).

Two-dimensional Electrophoresis of Denatured Enzyme—
The purified purine nucleoside phosphorylase which gave a single component on sodium dodecyl sulfate acrylamide gel electrophoresis was resolved into four components of the same molecular weight (subunits 1, 2, 3, 4) but different isoelectric points when analyzed by denaturing two-dimensional electrophoresis (33) as depicted in Fig. 4A. The apparent isoelectric points of the subunits 1 to 4 are 6.63, 6.41, 6.29, and 6.20, respectively. The correlation between the change of isoelectric point and ionic charge obtained by carbamylation (34) of the partially purified fraction from DEAE-chromatography of 0.2 ml of erythrocytes are visible in the two-dimensional electropherogram even after a 50-fold dilution (not shown). The correlation between the change of isoelectric point and ionic charge obtained by carbamylation (34) of the partially purified fraction from DEAE-chromatography of 0.2 ml of erythrocytes are visible in the two-dimensional electropherogram even after a 50-fold dilution (not shown).

The purified purine nucleoside phosphorylase subunits can be easily identified in the pooled DEAE-fractions eluted with 90 mM KCl and subsequently applied to the affinity column, as depicted in Fig. 4B. As can be seen in Fig. 4, C and D, the purification on DEAE-Sephadex A-50 does not seem to eliminate any major additional subunits of purine nucleoside phosphorylase. DEAE-fraction C (Fig. 4C) which was eluted at 50 mM KCl and prior to that DEAE-fraction used for the affinity chromatography, contains only the three most basic subunits (1, 2, 3); while DEAE-fraction D (Fig. 4D), which was eluted at 150 mM and subsequent to that DEAE-fraction used for affinity chromatography, contains only the three most acidic subunits (3, 4). When DEAE-fraction C and DEAE-fraction D were further purified and concentrated by affinity chromatography and then analyzed by two-dimensional chromatography, the former still revealed only subunits 1 to 3 while the latter contained detectable quantities of one and maybe two subunits more acidic than subunit 4, Fig. 4, E and F. The latter represent only 1 to 2% of the total phosphorylase protein.

Partially purified purine nucleoside phosphorylase from young and old erythrocytes separated by density on Ficoll (35) upon analysis by two-dimensional gel electrophoreco exhibited indistinguishable patterns of subunits. The abundance of purine nucleoside phosphorylase in human erythrocytes makes it possible to detect the enzyme protein molecules in a two-dimensional analysis of crude hemolysates (Fig. 5A). The assignment of purine nucleoside phosphorylase subunits in the two-dimensional electropherogram of the crude hemolysate can be verified by enrichment of the crude hemolysate with purified purine nucleoside phosphorylase as shown in Fig. 5B. The phosphorylase subunits are observed more distinctly in preparations purified by single-step DEAE-chromatography (Fig. 5C) or single-step affinity chromatography (Fig. 5D) from 0.2 to 0.5 ml of erythrocytes of normal individuals. Three of the phosphorylase subunits in the partially purified fraction from DEAE-chromatography of 0.2 ml of erythrocytes are visible in the two-dimensional electropherogram even after a 50-fold dilution (not shown).

Discussion

The use of formycin B, a competitive inhibitor ($K_i \sim 10^{-4} M$) of purine nucleoside phosphorylase (36), as an affinity ligand made it possible to purify to homogeneity purine nucleoside phosphorylase from human erythrocytes in two steps with an overall yield of 50%. The affinity chromatographic process gave a 100% yield and resulted in a 20-fold concentration of the enzyme. The purine nucleoside phosphorylase is judged pure by several criteria. (a) It exhibits a single component upon electrophoresis on acrylamide in the presence of sodium dodecyl sulfate. (b) The specific catalytic activity remained constant during the elution from the affinity chromatographic column and in a subsequent step. (c) When electrophoresed in native acrylamide gels of three different concentrations and (d) when isoelectric focused in acrylamide under native conditions, the catalytic activity of the purified purine nucleoside phosphorylase and the protein detected by staining coincide.

The specific catalytic activity of the purified purine nucleoside phosphorylase determined at 25° is only 57% of the value reported by Parks and colleagues (13, 14). However, enzyme inactivation cannot account for the observed difference since 92% of the activity loaded on the Sephadex A-50 was recovered in that step, and the recovery from the affinity chromatography was 100%. The specific catalytic activity of our crude hemolysate (0.0198 unit/mg) is nearly twice that reported by Kim et al. (13). Kim et al. used 4- to 6-week-old human blood while the blood used in our purification was freshly drawn.

The native molecular weights determined by the method of Hedrick and Smith (32) and the subunit molecular weight determined by 12 electrophoretic analyses on sodium dodecyl sulfate-acrylamide gels confirmed the trimeric structure of the native purine nucleoside phosphorylase from human erythrocytes. Substrate binding and genetic studies also support the conclusion that purine nucleoside phosphorylase exists natively as a trimeric structure (13, 21). On the basis of the fold purification and yields, it can be calculated that there are approximately 130 μg of purine nucleoside phosphorylase/ml of packed erythrocytes. Assuming a native molecular weight of 90,000, this corresponds to 1.44 nmol of purified purine nucleoside phosphorylase/ml of packed erythrocytes or 105 molecules/erythrocyte.

An interesting aspect of these studies is the two-dimensional gel electrophoretic pattern of purified human erythrocyte purine nucleoside phosphorylase. There are evident four major and one or two minor phosphorylase subunits each with the same molecular weight but different isoelectric points. The most alkaline subunit which is believed to be the primary one (35) was designated as subunit 1 and the more acidic components as 2, 3, and 4. On the basis of the correlation between isoelectric point and ionic charge of phosphorylase subunits, the subunits 2, 3, and 4 differ from the most alkaline subunit by 2, 3, and 4 negative charges, respectively. These additional negative charges could represent enzyme modification in the form of deamidation, phosphorylation, or acetylation. As explained above, appropriate controls indicate that no other major subunits present in the hemolysate were lost during the purification procedure.

The fact that the phosphorylase subunits can be partially separated by DEAE-Sephadex chromatography is a strong indication that the observed subunits are not an artifact of the two-dimensional electrophoretic analysis. Such an artifact could, for example, arise by carbamylation of the phosphorylase protein by cyanate during the electrophoresis in 9.5 M urea. Were the multiple subunits an artifact of the two-dimensional electrophoretic analysis, all of the enzyme preparations should exhibit the same subunit pattern.

Genetic evidence indicates that there is a single genetic locus in humans for purine nucleoside phosphorylase (21). Therefore, the differently charged subunits represent post-translational modification of a single polypeptide. The observed subunit alterations appear to be the result of specific in vivo modifications since cold storage of crude hemolysates.
or partially or completely purified enzyme preparations did not change the observed subunit pattern. The stoichiometry of the subunits is difficult to assess accurately since purification alters the relative quantities of the various subunits. Quantitative estimates based on two-dimensional gels of crude hemolysates or of phosphorylase purified by single-step affinity chromatography indicate that subunits 2 and 3 are present at equal concentrations, subunit 1 at one-half and subunit 4 at one-quarter of the concentrations of 2 and 3.

Human erythrocytic purine nucleoside phosphorylase can be resolved into at least seven isozymes on native starch gel electrophoresis (21). Isoelectric focusing in a column containing a sucrose gradient resulted in six peaks of enzymatic activity with isoelectric points ranging from 5.65 to 6.25; the pl values of 5.0 to 6.1 obtained by our studies differ from those obtained by Agarwal et al. (37). Individual isozymes cannot be distinguished on native acrylamide gel isoelectric focusing. Treatment with 4.5 \text{M} urea did not change the native isoelectric focusing pattern suggesting that the native pattern is not a result of binding of small molecules by the enzyme.

The complex patterns of isozymes observed upon electrophoresis or native isoelectric focusing of purine nucleoside phosphorylase can be explained by the subunit structure of the enzyme. Assuming that purine nucleoside phosphorylase is a trimer composed of four different subunits, the number of possible isozymes is 20. If the assigned charge differences of the subunits are correct, the number of isozymes with distinct charge differences is reduced to 12. Additional isozymes can arise by the participation of the minor subunits in the formation of isoenzymes and if the association of different subunits leads to conformational isomers.

The abundance of purine nucleoside phosphorylase in human erythrocytes makes it possible to detect purine nucleoside phosphorylase in a two-dimensional gel of a crude hemolysate. With the single-step partial purification by DEAE-chromatography described under “Experimental Procedures” from 1 ml of erythrocytes, as little as 0.3% of the normal level of purine nucleoside phosphorylase protein can be detected by the two-dimensional gel system. Thus screening for the presence of purine nucleoside phosphorylase subunit proteins in partially purified hemolysates by two-dimensional gel electrophoresis is as sensitive as antibody titration and is not dependent upon the maintenance of the antigenicity of the genetically altered protein. The fractionation process utilized prior to the two-dimensional analysis does not limit detection to proteins with minimally altered isoelectric points.

Two purine nucleoside phosphorylase-deficient patients and the relatives on one were screened by the two-dimensional gel electrophoretic technique and the results will be reported in a subsequent communication.1

REFERENCES
Purification and characterization of human erythrocyte purine nucleoside phosphorylase and its subunits.
V Zannis, D Doyle and D W Martin, Jr


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