Purine Nucleoside Phosphorylase from Human Erythrocytes

IV. CRYSTALLIZATION AND SOME PROPERTIES*

(Received for publication, July 29, 1968)

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SUMMARY

Purine nucleoside phosphorylase has been purified about 7,300-fold and crystallized from human erythrocytes (mol wt 81,000). The recrystallized enzyme exists in the form of needles and sometimes bundles of needles and has a specific activity of 96 \( \mu \text{M} \) units per mg of protein. A number of phenomena reported earlier for a less pure preparation of this enzyme are still seen with the crystalline enzyme. These include (a) substrate activation by inosine and (b) a constant ratio of reactivity with inosine and deoxyinosine. However, the ribosyl transfer reaction seems markedly decreased with the crystalline preparation. The effect of pH on maximal velocity suggests the occurrence of essential imidazole and sulfhydryl groups, whereas the effect of pH on the Michaelis constant suggests the existence of an essential ionizing group at about pH 7.2 in the free enzyme. Binding studies with hypoxanthine-8-\(^{14}\)C indicate that there are at least three binding sites per molecule of enzyme. When the commercially available bovine spleen purine nucleoside phosphorylase was compared with human erythrocytic enzyme, the two enzymes were found to be similar in molecular weight. However, they have different crystal structures and different specific activities, and in contrast to the behavior of the human enzyme, the spleen enzyme did not display the phenomena of substrate activation at high concentrations of inosine.

In recent publications, this laboratory reported the purification and some of the properties of human erythrocytic purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) (1-3). The best preparations of enzyme had specific activities of about 80 \( \mu \text{M} \) units per mg of protein and appeared close to homogeneity by Sephadex chromatography. A kinetic analysis indicated that the reaction follows an “ordered Bi-Bi mechanism” (4), with the nucleoside the first substrate to add and the base the last product to leave the enzyme surface. The ratio of the reaction velocities with inosine and deoxyinosine as substrates remained constant throughout purification as did the ratio between a slow ribosyl transferase activity and the phosphorolytic activity. Substrate activation was seen at high concentrations of nucleoside, suggesting that possibly an isozyme with a high Michaelis constant is present or that the enzyme is multivalent with cooperative interaction between the active sites. Therefore, it was important that the enzyme be purified further to a state of homogeneity.

It has been possible to crystallize the enzyme at a specific activity of 96 \( \mu \text{M} \) units per mg of protein. The present report is concerned with studies of crystalline human erythrocytic purine nucleoside phosphorylase and makes comparisons with the commercially available crystalline purine nucleoside phosphorylase from bovine spleen recently studied by Krenitsky (5). A preliminary report of this work has been presented.2

EXPERIMENTAL PROCEDURE

Materials—DEAE-cellulose (Cellex D), deoxyinosine, dithiothreitol (Cleland’s reagent), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, Tris, pyruvate kinase (rabbit muscle, crystalline), the sodium salts of ADP, ATP, and DPNH, and trisodium phosphoenolpyruvate were obtained from Sigma. Hypoxanthine and purine nucleoside phosphorylase (bovine spleen, crystalline, specific activity 22 \( \mu \text{M} \) units per mg of protein) were the products of Boehringer Mannheim, and xanthine oxidase was purchased from Worthington. Inosine, guanine, and guanosine were obtained from P-L Biochemicals. Sephadex was obtained from Pharmacia, and Whatman chromatography cellulose powder (standard grind) from H. Reeve Angel Company. Crystalline bovine albumin was a product of the Armour Pharmaceutical Company. Hypoxanthine-8-\(^{14}\)C was obtained from Schwarz BioResearch. Calcium phosphate gel was prepared according to the method of Tsurbi and Hudson (6). Human blood was kindly supplied by Dr. Jacob Dyckman, Director of the Blood Bank of the Miriam Hospital in Providence, Rhode Island.

Radioactivity was measured in a Packard TriCarb scintillation spectrometer, model 3600, in Bray’s solution (17).

Methods—The assay method, which is an adaptation of the coupled xanthine oxidase procedure of Kalckar (8), the definition of the enzyme unit and of specific activity, and protein determination were as described earlier (1).

Acrylamide gel electrophoresis was performed by a modification of the method of Clark (9). Aliquots of the reconstituted enzyme (50, 25, 15, and 10 μg) were applied on the top of the gel in 10% sucrose solution. Electrophoresis was performed in Tris glycine buffer (pH 8.3) for 30 min at 700 volts and 5 ma per tube. After the run, the protein was stained for 1 hour with 0.25% Amido schwarz in 7% (v/v) acetic acid. Excess dye was removed by washing with water and 7% acetic acid and finally by destaining electrophoretically in 7% acetic acid at a current of about 10 ma per tube. The gel was stored in 7% acetic acid.

RESULTS

Crystallization of Purine Nucleoside Phosphorylase from Human Erythrocytes—By the use of a procedure essentially similar to that described earlier (1), it has been possible to purify the purine nucleoside phosphorylase from 12 pints of human blood to a specific activity of 70 μm units per mg of protein with an over-all recovery of 36% of the enzyme. The enzyme solution carried through the Sephadex chromatography step as described earlier (1) (4900 units; specific activity, 70 μm units per mg) was brought to 70% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and diluted with water to a protein concentration of about 10.6 mg per ml. Saturated ammonium sulfate solution was added to about 35% saturation whereupon the solution became slightly turbid. The turbidity was removed by centrifugation at 10,000 × g for 30 min, and the amorphous precipitate was discarded. To the supernatant fluid more ammonium sulfate solution was added to bring the concentration to about 40% saturation whereupon slight turbidity again appeared. The solution was left overnight at 4°C. About 60% of the enzyme precipitated as fine needles or as bundles of needles (Fig. 1a), and the specific activity increased to 94.4. An aliquot was saved for seeding later preparations.

The crystals of enzyme were collected by centrifugation at 10,000 × g for 10 min, dissolved in a small volume of water, and adjusted to 40% saturation with saturated ammonium sulfate, and a few seed crystals from the preceding step were added. Again needle-shaped crystals appeared within 30 min (Fig. 1b). After about 12 hours at 4°C, approximately 95% of enzyme was recovered as crystals and the specific activity was 96. Fig. 1 also includes a photograph of the crystalline bovine spleen purine nucleoside phosphorylase. In contrast to the human erythrocyte enzyme, the crystals of the bovine spleen enzyme occur in flat plates (Fig. 1c).

Properties of Recrystallized Enzyme—Acrylamide gel electrophoresis revealed a single major zone of protein with a slow moving zone of a trace protein (which was estimated as less than 1% of the total) near the cathode, which was visible in the 50-μg sample. Also, symmetrical and coincident peaks of protein and enzyme activity were obtained by chromatography on Sephadex G-100. Thus, by two criteria, acrylamide gel electrophoresis and molecular sieve chromatography, the enzyme appears essentially homogeneous with a trace contaminant of less than 1% seen by electrophoresis.

The estimation of molecular weight of about 81,000 by the method of Andrews (10) confirmed the earlier results (1). The molecular weight of the bovine spleen enzyme was similar to that of the human erythrocyte enzyme.

In view of the possibility that a conformational change takes place in the enzyme molecule in the presence of high concentrations of nucleoside (1, 2), an experiment was performed to test whether there is a difference in the molecular volume of the enzyme in the presence and in the absence of inosine. There was no difference in the elution volume of the enzyme when filtered through the Sephadex G-200 column in the presence of 0.1 M inosine and in the absence of inosine.

The stability of the crystalline enzyme to variations in protein concentration and ionic strength and to the presence of thiold reagents and the specificity toward inosine and deoxyinosine were essentially the same as reported earlier for the less pure enzyme (1). However, the slow ribosyl transfer activity seemed markedly decreased in the crystalline preparation. This apparent loss of activity has not been explained and is the subject of current studies.

Incubation of solutions of crystalline enzyme at various temperatures for 15 min in 0.1 M Tris acetate, pH 7.5, at the protein concentration of 40 μg per ml, resulted in 50% inactivation at about 57°C. In contrast to the report of Devenedier and Gould (11), reactivity to inosine and deoxyinosine decreased in parallel. The enzyme was stable at pH values 6.2 to 10.0 for 5 min at room temperature.

The ultraviolet absorption spectrum of the crystalline enzyme was not exceptional and peaked at 279 μm in 0.1 M Tris acetate, pH 7.5. The molar extinction coefficient (εmax) was calculated to be 8.91 × 104 at pH 7.5.

Substrate Activation at High Concentrations of Inosine—In the earlier studies, activation by high nucleoside concentrations raised the possibilities of the occurrence of an isoenzyme with a high Michaelis constant. However, the same substrate activation phenomenon was seen with the recrystallized human enzyme (Fig. 2), which makes the occurrence of an isoenzyme unlikely. In contrast to the behavior of the crystalline human enzyme, the crystalline bovine spleen enzyme did not display substrate activation at high concentrations of inosine (Fig. 2).

Effect of pH on Kinetic Properties—Km and Vmax values for inosine and deoxyinosine were determined by extrapolation of the linear portions of the Lineweaver-Burk plots (12) over pH values from 5.4 to 10.0. The results have been plotted according to the method of Dixon (13) (Fig. 3). In the plot of Vmax against pH, the downward breaks suggest that there are ionizing groups in the enzyme-substrate complex which have pK values of about pH 6.4 and 8.3. The break at pH 6.4 would be consist-

![Fig. 1. Crystals of human erythrocytic purine nucleoside phosphorylase in ammonium sulphate solution. a, bundles (× 100); b, needles (× 1000); c, crystals of bovine spleen enzyme (× 400).](http://www.jbc.org/issue/17/2/264/supplemental/141.png)
ent with the ionization of a histidine group in the enzyme-substrate complex. In the plot of $pK_m$ against $pH$, a concave upward break at about $pH$ 8.1 is consistent with the occurrence of an ionizing group in the enzyme-substrate complex and coincides closely with a similar break in the $V_{max}$ against $pH$ plot. It is possible that a sulfhydryl group in the enzyme-substrate complex is responsible for these findings. The convex curvature at $pH$ 7.2 could be produced by an ionizing group in the free enzyme or in the substrate. Since there is not a $pK_a$ for either inosine or deoxyinosine in the region of this $pH$, it seems likely that this is due to the ionization of a group on the free enzyme. It should be noted that in these experiments the phosphate concentration was held constant at 50 mM which is more than 10 times greater than the $K_m$ for phosphate over this $pH$ range. Therefore, although phosphate has a $pK_a$ in this range, it is unlikely that inorganic phosphate plays a role in this finding. In similar studies with orthophosphate over a wide range of $pH$ values, unusual kinetic behavior was observed. The Lineweaver-Burk plots were linear in the region of $pH$ 7.0, but at $pH$ values of 6.0 and below, substrate activation was observed, whereas at $pH$ values above 8.0 substrate inhibition was seen at higher concentrations of phosphate. These unusual observations are under detailed study.

**Number of Binding Sites**—An estimation of the number of binding sites per molecule of purine nucleoside phosphorylase was made by a gel filtration technique similar to that used by Custrecassas, Fuchs, and Anfinsen (14). In this procedure, known amounts of crystalline enzyme were filtered through a Sephadex G-100 column. The buffer solution with which the column was equilibrated and eluted contained a constant small concentration of hypoxanthine-$8^3$H. Fractions were collected and the amounts of enzyme protein and hypoxanthine in each tube were estimated. The amount of hypoxanthine which formed a complex with enzyme was determined by the increase in radioactivity in the tubes containing protein. Several experiments were performed in a similar manner at different concentrations of hypoxanthine, and in each case the number of molecules of hypoxanthine bound per molecule of enzyme was determined. A straight line was obtained when $\bar{r}$ was plotted versus $e/d$ according to the Krone equation (15).

$$\bar{r} = n - k_d (\bar{e} / \bar{d})$$

where $\bar{r}$ is the number of moles of hypoxanthine bound per $81,000$ g of enzyme (molecular weight), $\bar{e}$ is the concentration of free hypoxanthine, $n$ equals $\bar{e}$ when $d$ equals infinity, and $k_d$ is the dissociation constant of the enzyme-substrate complex (i.e. enzyme-hypoxanthine). Upon extrapolation to infinite substrate concentration, this line intersected the vertical axis at about 3.0, which suggests that there are at least three binding sites per molecule of enzyme. With the present technique it was not possible to use high concentrations of hypoxanthine. Deviations from linearity which might have occurred due to conformational effects at high substrate concentration could not be observed. It should be noted that from the slope of this line the dissociation constant of the hypoxanthine-enzyme complex can be determined. This value from the graph was $1.6 \times 10^{-4} M$. We have not determined the $K_m$ of hypoxanthine with this enzyme, but the $K_i$ of guanine is in this order of magnitude, $5 \times 10^{-4} M$ (2).

**Discussion**

This paper describes the crystallization of human erythrocytic purine nucleoside phosphorylase. When the crystalline enzyme was compared with the partially purified preparation described earlier (1, 2), the stability properties, molecular weight, ratios of reactivity to inosine and deoxyinosine, and the occurrence of substrate activation at high concentrations of nucleoside remained unchanged. However, upon crystallization a marked
apparent decrease was observed in the ribosyl transfer reaction in the absence of orthophosphate, which requires further study.

The occurrence of substrate activation at high concentrations of nucleoside with the recrystallized enzyme appears to rule out the possible occurrence of an isoenzyme as an explanation of this phenomena. In the earlier studies it was suggested that nucleoside phosphorylase is multivalent with cooperative interaction between active sites (1, 2). This hypothesis is supported by the present studies in which there appear to be at least three hypoxanthine binding sites per molecule of enzyme. The techniques used here were not sufficiently sensitive to reveal high orders of binding sites, and a conclusion on the absolute number of subunits per molecule of enzyme must of necessity be deferred for the completion of further studies, such as attempts to dissociate the enzyme into its constituent subunits, the performance of end group analyses, and determination of the amino acid composition. Before these studies can be undertaken it will be necessary to isolate larger amounts of crystalline enzyme.

A major difference in the kinetic behavior of the human and bovine enzymes is that with the human enzyme, competition was observed between the purine base and the nucleoside (2), whereas with the bovine spleen enzyme noncompetitive inhibition was observed (5). Also with the human enzyme the inhibition between phosphate and ribose-1-phosphate was noncompetitive (2), while with the bovine spleen enzyme there appeared to be a competitive relationship (5). In the case of the human erythrocytic enzyme, initial velocities were used, and the reaction investigated was the phosphorolysis of inosine and deoxyinosine (2). In contrast with the bovine spleen enzyme, the synthesis of inosine was investigated by using 15-min incubation periods (5). It is possible that the discrepancies in the results with the two enzymes are due to the different methods of study. Comparative initial velocity and product inhibition studies should be performed with the use of identical techniques. However, it should be noted that the two enzymes display distinct differences in properties. Although the molecular weights are similar (about 81,000) the specific activity of the crystalline bovine spleen enzyme is about one-third that of the human erythrocytic enzyme. The crystal structures are quite different (Fig. 1), and in experiments performed simultaneously with the human erythrocytic and bovine spleen enzymes, substrate activation by inosine occurred with the former but not with the latter (Fig. 2). It will be of interest to make further comparisons of these two enzyme preparations since an explanation of the differences in the substrate activation and kinetic behavior may yield valuable insights into the nature of conformational changes which may occur.

Acknowledgments—We wish to acknowledge the interest and advice of Drs. Sungman Cha, Byung Kyu Kim, and Mrs. Margaret Sheen. We wish to thank Mrs. C. A. Weissinger for taking the photomicrographs.

REFERENCES


It may be noted that when purine nucleoside phosphorylase was isolated from freshly drawn human red blood cells, substrate activation was observed which was identical to that seen with the crystalline enzyme. This indicates that the substrate activation phenomenon was not the result of an effect on the enzyme which occurred due to aging of the blood prior to enzyme isolation.