Identification of Dihydropteridine Reductase in Human Platelets

By Herbert T. Abelson, Carolyn Gorka, and G. Peter Beardsley

Normal human platelets were shown to contain the enzyme dihydropteridine reductase. The enzyme was not found in a variety of other cells of hematogenous origin. Partial purification and kinetic and physical data indicated that the platelet enzyme is similar to that previously characterized from liver. Dihydropteridine reductase is important for the regeneration of tetrahydrobiopterin, a required cofactor in hydroxylation reactions involved in biogenic amine formation. The presence of the enzyme may indicate that some synthesis de novo of serotonin and/or catecholamines occurs in platelets, as opposed to a purely storage and transport function. In addition, screening for hyperphenylalaninemia due to dihydropteridine reductase deficiency may become feasible by assaying platelets for enzyme activity.

Dihydropteridine reductase is important for the regeneration of tetrahydrobiopterin, a cofactor in the enzymatic conversion of phenylalanine to tyrosine, tyrosine to DOPA, and tryptophan to 5-hydroxytryptophan (5-HT). Platelet storage granules contain high concentrations of 5-HT and other biogenic amines that are actively transported into platelets from the blood. This finding, in addition to the previous inability to detect the requisite synthetic enzymes in platelets, led to the generally accepted conclusion that platelet 5-HT and catecholamines are not synthesized in situ.

However, one of the synthetic enzymes, tryptophan 5-hydroxylase, has now been identified in human platelets at levels comparable to those in some portions of the nervous system. The conversion of tryptophan to 5-hydroxytryptophan, the first step in 5-HT formation, may therefore occur in platelets. This hydroxylation reaction has an absolute requirement for tetrahydrobiopterin as a cofactor.

Tetrahydrobiopterin levels are maintained primarily by the reduction of quinonoid dihydrobiopterin to tetrahydrobiopterin by dihydropteridine reductase.

Dihydropteridine reductase was previously found to be absent from serum, erythrocytes, and leukocytes. We now report the presence and partial characterization of dihydropteridine reductase from normal blood platelets as well as the absence of this enzyme in a variety of other cells of hematogenous origin. Therefore another important enzyme in the synthetic pathways for 5-HT and catecholamines has been identified in platelets.
DIHYDROPTERIDINE REDUCTASE IN PLATELETS

MATERIALS AND METHODS

NADPH, NADH, catalase, and horseradish peroxidase were obtained from Sigma Chemical, St. Louis. MTT [3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazoliumbromide] and DMPH₄ (6,7-dimethyl-5,6,7,8-tetrahydropterin) were purchased from Aldrich Chemicals (Milwaukee, Wis.).

Enzyme preparations: platelets. Freshly prepared platelet concentrates or platelets greater than 48 hr old (stored at room temperature) were found to be equally good for the isolation and recovery of dihydropteridine reductase activity.

To prepare concentrated platelet suspensions, platelet-rich plasma was centrifuged twice at 800 g for 5 min at 4°C to sediment red and white blood cells. The platelet-rich supernatant was then centrifuged at 5000 g for 30 min at 4°C to pellet the platelets. The pellet was taken up in 5 vol 0.03 M acetic acid and sonicated for 30 sec at setting 4 on a Branson model W 185 sonicator.

The sonicate was then centrifuged at 12,000 g for 10 min at 4°C to pellet membranes. The supernatant was removed, neutralized to pH 7.4 with 0.1 N KOH, and 35 g ammonium sulfate per 100 ml was added. The mixture was stirred for 1 hr at 4°C and then centrifuged at 12,000 g for 10 min at 4°C and the precipitate discarded. To the supernatant, an additional 10 g ammonium sulfate per 100 ml of original volume was added. The mixture was stirred at 4°C for 1 hr and then centrifuged again at 12,000 g. The pellet was taken up in 0.01 M Tris-HCl pH 7.8 and adjusted to a final volume equal to 20% of the original extract volume. This solution was dialyzed overnight against 0.01 M Tris-HCl pH 7.8. The dialysate was loaded onto a DEAE-cellulose column previously equilibrated with 0.01 M Tris-HCl pH 7.8 and washed overnight with at least five bed volumes of the same buffer. The enzyme was eluted with a 500-mI gradient of 0-0.5 M KCl in 0.01 M Tris-HCl pH 7.8. Then 5-mI fractions were collected, and absorbance at 280 nm was monitored. Aliquots of each fraction were assayed for enzyme activity as below, and peak fractions were pooled.

Enzyme preparations: others. Crude extracts were also prepared from red blood cells, Ficoll-Hypaque-separated peripheral blood lymphocytes before and after phytohemagglutinin (PHA) stimulation, cultured human HSB2 lymphoblasts, and cells from patients with acute myelocytic leukemia (myeloblasts) and chronic myelocytic leukemia (mature granulocytes). Briefly, cells were suspended in 50 mM Tris-HCl pH 7.5 and freeze-thawed three times followed by sonication for 30 sec as above. After centrifugation at 12,000 g for 10 min at 4°C, the extracts were assayed for dihydropteridine reductase.

Dihydropteridine reductase assay. Enzyme assays were performed as previously described.¹ For DMPH₄ kinetic studies, each reaction contained Tris-HCl (pH 7.2) 50 μmoles, NADH 0.1 μmoles, H₂O₂ 0.9 μmoles, peroxidase 8 μg, dihydropteridine reductase 50 μg, and various amounts of DMPH₄. For NADH kinetic studies, each reaction contained 0.01 μmoles DMPH₄ and varying amounts of DMPH₄. The concentrations of other components remained the same. The reaction volume totaled 1 ml. Reactions were monitored continuously at 340 nm against a blank that lacked enzyme. No reaction occurred in either system when bovine serum albumin was substituted for dihydropteridine reductase.

Dihydrofolate reductase assay was performed spectrophotometrically as previously described.²

Disc gel electrophoresis. Electrophoresis on sodium dodecyl sulfate (SDS)–containing gels was by the method of Steck.¹⁰ Nondenaturing gel electrophoresis was performed according to the method of Ornstein and Davis.¹¹ To stain for protein, gels were left overnight in 0.05% Coomassie Brilliant Blue and then destained in 7.5% acetic acid. Staining for dihydropteridine reductase activity in situ was as previously described¹² or using a mixture containing (in 5 ml) Tris-HCl (pH 7.5) 240 μmoles, DMPH₄ 0.5 μmoles, MTT 5 μmoles, and NADH 5 μmoles. Gels were treated with this mixture at room temperature for 15 min. DMPH₄ was generated from DMPH₄ in 5 min at room temperature in a mixture containing (in 0.2 ml) H₂O₂ 10 μmoles, DMPH₄ 50 μmoles, and peroxidase 80 μg. Catalase 1250 units was then added and the reaction mixture placed on ice.

RESULTS

Partial purification of platelet dihydropteridine reductase. The partial purification of enzyme from 12 units of platelets is summarized in Table I. This procedure resulted in a 25-fold increase in specific activity. The actual specific activity achieved, 0.076 units/mg, is much lower than the 2.51 units/mg reported
Table 1. Partial Purification of Human Platelet Dihydropteridine Reductase (12 Units Platelets)

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total Activity (Units)</th>
<th>Total Protein (mg)</th>
<th>Sp. Act (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>17.0</td>
<td>1.68</td>
<td>552.5</td>
<td>0.003</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fractionation</td>
<td>3.4</td>
<td>0.98</td>
<td>54.4</td>
<td>0.018</td>
<td>58</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peak tubes</td>
<td>10</td>
<td>0.55</td>
<td>7.2</td>
<td>0.076</td>
<td>33</td>
</tr>
</tbody>
</table>

*One unit of enzyme activity is taken as that amount of enzyme that will catalyze the oxidation of 1 \( \mu \)mole reduced pyridine nucleotide at 25°C in 1 min.

for enzyme from human liver purified in the same fashion. We also repeated the human liver findings using autopsy material that had been stored at -70°C, attaining a final specific activity of 1.7 units/mg.

**Enzymatic properties of human platelet dihydropteridine reductase.** The enzyme used for these studies was purified through the DEAE-cellulose step as above. Further purification resulted in loss of enzyme activity. The initial rate of the reaction was proportional to the amount of protein added between 25 and 100 \( \mu \)g/ml.

Figures 1 and 2 show double-reciprocal plots of \( V_{\text{max}} \) versus the substrate concentration for DMPH\(_4\) and NADH using human platelet dihydropteridine reductase. These data are summarized in Table 2 along with kinetic data from human liver.

When NADPH was substituted for NADH, the rate of reaction with platelet dihydropteridine reductase became too low to measure accurately. Liver enzyme also displays similar cofactor specificity. No dihydrofolate reductase activity was identifiable in the crude or partially purified platelet preparations.

We also assayed crude extracts from a variety of other cells for dihydropteridine reductase activity, including red blood cells, Ficoll-Hypaque-separated peripheral blood lymphocytes before and after PHA stimulation, cultured human HSB2 lymphoblasts, myeloblasts from a patient with acute myelocytic leukemia, and mature granulocytes from a patient with chronic myelocytic leukemia. None of these extracts contained detectable dihydropteridine reductase activity, ruling out the possibility that the low level of dihydropteridine reductase activity in platelets resulted from contamination with other cell types.

![Fig. 1. Double-reciprocal plot to determine \( K_d(DMPH_4) \) of dihydropteridine reductase from normal peripheral blood platelets. Conditions are given in Materials and Methods. Straight line was drawn by the method of least squares.](image-url)
After electrophoresis on 7.5% nondenaturing gels, staining in situ for dihydropyridine reductase activity was attempted. However, reaction product was always found in more than one band, limiting the utility and specificity of this reaction in our hands.

Physical properties of platelet dihydropyridine reductase. Enzyme purified through the DEAE-cellulose step was run on 7% SDS polyacrylamide gels to determine molecular weight. Molecular weight standards used were cytochrome C, myoglobin, chymotrypsinogen A, ovalbumin, BSA, and phosphorylase A. On multiple determinations, platelet dihydropyridine reductase gave a band corresponding to a molecular weight of 25,000–28,000 daltons. If samples were boiled in SDS without 2-mercaptoethanol, a band was seen at 45,000 daltons. Small amounts of hemoglobin were present in the platelet preparations.

DISCUSSION

We have shown that human peripheral blood platelets contain low levels of dihydropyridine reductase activity. This enzyme does not appear to be present in other elements of blood, as evidenced by our studies of a wide variety of cell types ranging from lymphoblasts and myeloblasts to mature erythrocytes, lymphocytes, and granulocytes. Other workers also reported erythrocytes, lymphocytes, and unfractionated leukocytes as well as serum to be devoid of dihydropyridine reductase activity. Thus among the formed elements of blood, dihydropyridine reductase activity appears to be unique to the platelet. The platelet enzyme has similar physical and kinetic properties to that reported for liver. In our hands, staining in situ for dihydropyridine reductase was not specific. This may be due to

Table 2. Summary of Kinetic Data for Human Platelet and Human Liver Dihydropyridine Reductase

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>DMPH₄ (μM)</th>
<th>Kᵣ (μM)</th>
<th>Vₘₐₓ (μmol/min/mg)</th>
<th>DMPH₄ (μM)</th>
<th>Kᵣ (μM)</th>
<th>Vₘₐₓ (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human platelets</td>
<td>10</td>
<td>12</td>
<td>0.09</td>
<td>100</td>
<td>16</td>
<td>0.30</td>
</tr>
<tr>
<td>Human liver</td>
<td>100</td>
<td>3.3</td>
<td>1.47</td>
<td>100</td>
<td>3.3</td>
<td>1.47</td>
</tr>
</tbody>
</table>
the production of reduced MTT in the reaction mixture, which nonspecifically stains proteins in the gel.

As mentioned above, dihydropteridine reductase is important for the regeneration of tetrahydrobiopterin from quinoid dihydrobipterin. Tetrahydrobipterin may also be produced by the reduction of 7,8-dihydrobipterin by dihydrofolate reductase.7,14 Both reactions probably contribute to the tetrahydrobipterin pool in liver and brain; however, the dihydropteridine reductase pathway must predominate in platelets, since dihydrofolate reductase activity does not appear to be present.

Tryptophan hydroxylase was previously identified in human platelets at levels comparable to those found in all regions of mammalian brain studied with the exception of the pineal.6 Other investigators, however, identified tryptophan hydroxylase in the nucleated platelets of domestic fowl but could not detect activity in either human or rabbit platelets.15 The finding of dihydropteridine reductase in platelets shows that another biochemical constituent necessary for the de novo synthesis of serotonin and/or catecholamines is present. It would seem reasonable to reserve final judgement on the origin of some platelet serotonin and catecholamines until the synthesis of these compounds in both platelets and megakaryocytes has been studied directly. A system for culturing guinea pig megakaryocytes was recently described16 and may allow this question to be settled.

Platelets accumulate, store, and release biogenic amines in a fashion similar to monoamine-containing neurons4 which led to the study of platelets as models for nervous tissue. The similarity was flawed in that platelets were not previously thought to have the capacity to synthesize biogenic amines. As suggested above, this may not be the case, and thus platelets may more closely resemble neurons than previously supposed.

In addition, the presence of dihydropteridine reductase in platelets may help to identify nonclassical forms of phenylketonuria. Hyperphenylalaninemia without phenylalanine hydroxylase deficiency is now recognized in several variants,8,17-20 some of which involve absence of dihydropteridine reductase activity. Low concentrations of 5-HT and dopamine, which require tetrahydrobipterin in their synthesis, have been found in the cerebral cortex of a patient with dihydropteridine reductase deficiency.20 The present identification of dihydropteridine reductase activity in normal platelets may allow for enzyme screening from peripheral blood, thereby obviating the need for liver biopsy or culture techniques for diagnosis of at least one of the variants.8 Study of platelet granules and platelet function in these patients would be of interest since several platelet dysfunction syndromes involving deficiencies of platelet biogenic amines have been described.21

REFERENCES
5. Zucker MB, Borelli J: A survey of some platelet enzymes and functions: The platelets as the source of normal platelets and platelet function in these patients would be of interest since several platelet dysfunction syndromes involving deficiencies of platelet biogenic amines have been described.21

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