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PURIFICATION OF 6-PYRUVOYL-TETRAHYDROPTERIN SYNTHASE FROM HUMAN LIVER

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The enzyme which catalyzes the first step in the conversion of dihydroneopterin triphosphate to tetrahydrobiopterin has been purified approx. 40,000-fold from human liver to apparent homogeneity. The enzyme has a native molecular weight of ~83,000 and consists of four identical subunits, each of which has a molecular weight of ~19,000. It contains carbohydrates and is remarkably stable to heat treatment. In the presence of purified sepiapterin reductase, Mg^{2+}, and NADPH, this enzyme catalyzes efficiently the formation of tetrahydrobiopterin from dihydroneopterin triphosphate. This indicates that these two proteins are sufficient for the overall conversion.

One of the key enzymes involved in the biosynthesis of BH_4[1], the cofactor for phenylalanine, tyrosine, and tryptophane hydroxylases (1), has been proposed to catalyze the conversion of H_2NTP to a metastable intermediate, 6-pyruvoyl-tetrahydropterin (PTP), and has therefore been named 6-pyruvoyl-tetrahydropterin synthase. Genetic deficiency of PTPS in man induces hyperphenylalaninemia (2) and a low level of neurotransmitters, such as catecholamine and indolamine. Recently we have shown that this enzyme catalyzes the elimination of triphosphate from H_2NTP (3), and we have reported its partial purification from human liver, as well as some of its properties. Similar enzymes have been partially purified from other sources (4,5,6), however, in most experiments involving formation of BH_4 from H_2NTP the enzyme preparation

[1] Abbreviations and trivial names: BH_4, tetrahydrobiopterin; H_2NTP, dihydroneopterin triphosphate; PTP, 6-pyruvoyl-tetrahydropterin; PTPS, 6-pyruvoyl-tetrahydropterin synthase; DTE, dithioerythritol; KP, potassium phosphate (buffer); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

used was not homogeneous. The use of a homogeneous enzyme is necessary in order to determine whether a single enzyme catalyzes both the elimination of triphosphate from H_2NTP as well as the internal oxidoreduction leading to PTP. In this paper we describe the purification of PTPS to apparent homogeneity from human liver, as well as some of its properties.

MATERIALS AND METHODS

Materials. Human liver was obtained from traffic accident victims and was frozen to -70°C within 30 min after death. H_2NTP was prepared from GTP enzymatically (7). BH_4 was purchased from Dr. B. Schircks Laboratories, Buechstrasse 17a, 8645 Jona, Switzerland. Hydroxyapatite was obtained from Bio-Rad. Ultrogel AcA 44 and AcA 34 were from LKB. DEAE-Fractogel 650 S and Fractogel TSK HW-50(S) were from Merck, Darmstadt, FRG. Sepiapterin reductase was prepared from human liver as previously described (3). All other chemicals were of reagent grade from commercial sources.

Activity determinations. The enzyme activity was determined by measuring the production of BH_4 with HPLC (electrochemical detection) (8). The standard reaction mixture contained the following components: Tris-HCl buffer, pH 7.4, 100 mM; $MgCl_2$, 8mM; DTE, 10 mM; NADPH, 1 mM; sepiapterin reductase, ca. 2mU (9); H_2NTP, 16 µM and the appropriate amount of PTPS in a final volume of 125 µl. After incubation at 37°C for 60 min, the reaction was stopped by adding 25 µl of 0.2 M EDTA and 10 µl of the solution was injected into the HPLC system. One unit of PTPS activity was defined as the amount which produced 1 pmole of BH_4 per min under the above conditions.

Procedure for the purification of PTPS. All procedures were carried out at 4°C. Human liver (250 g) was thawed, cut into small pieces, and homogenized in a Virtis-45 homogenizer for 3 min with 2 vol of 0.01 M KP buffer, pH 7.0 at 1/2 full speed, and then with another 2 vol of the same buffer for 3 min at 2/3 full speed. The homogenate was centrifuged at 27,000 g for 60 min and the supernatant filtered through cheese cloth to remove fat. Solid ammonium sulfate was then added to 35% saturation, stirred for 40 min, and the precipitate removed by centrifugation at 11,000 g for 60 min. The supernatant was again filtered through cheese cloth to remove the last residues of fat, and solid ammonium sulfate was added to 50% saturation. The mixture was stirred for 40 min, and the precipitate was collected by centrifugation at 11,000 g for 60 min. The protein was dissolved with 80 ml of 0.01 M KP buffer, pH 6.0 and dialyzed overnight against 2 changes of 10 l of the same buffer. The ammonium sulfate fractions obtained from 2 ammonium sulfate fractions (i.e., starting from a total of 500 g liver) were centrifuged at 27,000 g for 60 min in order to remove precipitated protein. The supernatant was applied to a hydroxyapatite column (5 x 45 cm) equilibrated with 0.01 M KP buffer, pH 6.0. The column was washed with 300 ml of the buffer (80 ml/h); a linear gradient from 1000 ml 0.01 M KP buffer, pH 6.0, to 1000 ml 0.2 M KP buffer, pH 6.0, was applied. The fractions containing PTPS activity were combined, concentrated (Amicon PM 10) to about 50 ml, and heated for 5 min at 80°C. The obtained suspension was cooled in an ice bath for 3 min and centrifuged at 17,000 g for 30 min. The supernatant was applied to an Ultrogel AcA 44 column (5 x 91 cm) equilibrated with 0.02 M KP buffer containing 0.2 M KCl, and eluted with the same buffer. Combined active fractions (50 ml/h, 10 ml/fraction) were concentrated (Amicon PM 10) to ca. 8 ml and dialyzed overnight against 2 changes of 5 l 0.02 M PK buffer, pH 7.0. The solution was applied to a DEAE-Fractogel 650 S column (2.6 x 34 cm) equilibrated with the dialysis buffer, and the column was then washed with 80 ml of this buffer. The proteins were eluted with 800 ml of a

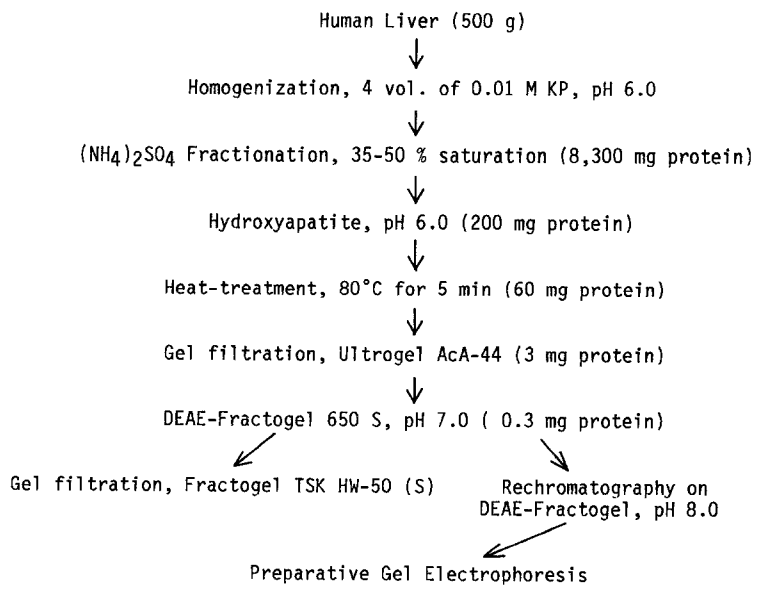
linear gradient of KCl (0-0.4 M) in 0.02 M KP buffer, pH 7.0 (10 ml fractions, 40 ml/h). The combined active fractions were concentrated (Amicon PM 10) and dialyzed overnight against 2 changes of 5 l 0.01 M KP buffer, pH 7.0. The purified enzyme is stable for several months at -20°C.

The protein was determined by the method of Lowry et al. by using bovine serum albumin as the standard (10). PAGE was performed by the methods of Davis (11) and PAGE in the presence of SDS was carried out as described by Laemmli (12); the proteins on the gels were visualized with a Bio-Rad silver stain kit. The carbohydrate was visualized on the gels according to the method of Zacharius et al. (13).

RESULTS AND DISCUSSION

The procedure for the purification of PTPS is summarized in Scheme 1, the sequence of steps shown was worked out in partial modification of the procedure published earlier (3), and according to the following criteria: The hydroxyapatite column chromatography was found to be essential since it provides an excellent separation of PTPS from sepiapterin reductase, and also allows a recovery of the second enzyme. Heat-treatment, a crucial step due to its effectiveness, and the little loss of activity accompanying it, could not be performed at an earlier stage.

The preparation obtained after DEAE-Fractogel chromatography was found by PAGE analysis to consist of a major band (80-90% of the total protein



Scheme 1. Procedure for the Purification of PTPS.

content), and of two minor bands. The specific activity of the enzyme at this purification step had been increased to about 40,000-fold, the amount of total protein was 0.3 mg (from 500 g human liver) corresponding to an overall recovery of 33% of the original activity. For final purification of PTPS a chromatography on CM-Fractogel at pH 6.0 was attempted since Masada et al. (6) used this ion-exchanger to purify PTPS from fat bodies of silkworm. However, in our hands, all the activity was lost during this process. Attempts to further purify PTPS by conventional chromatographic steps were mainly unsuccessful, the two protein impurities comigrating in all cases. PTPS was therefore subjected to preparative PAGE (7% T), and the enzyme was eluted by homogenization and extraction of the main band from the gel. In this experiment the activity corresponded very well with the main protein band observed by staining as described in the M&M section. This fraction, when resubjected to SDS-PAGE yielded a single band corresponding to a molecular weight of approx. 19,000 D (Fig. 1). The molecular weight determination

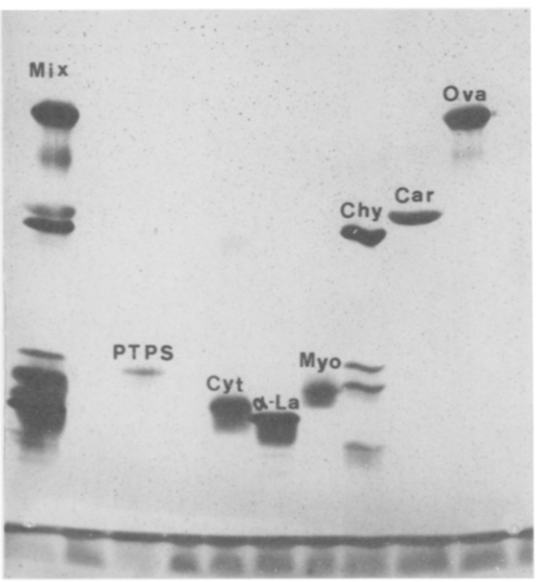


Fig.1. SDS-PAGE with 12.5% polyacrylamide. Ova, ovalbumin (MW = 45,000); Car, carbonic anhydrase from bovine erythrocytes (29,000); Chy, chymotrypsinogen A from bovine pancreas (25,700); Myo, myoglobin from equine skeletal muscle (17,800); α-La, α-lactalbumin from bovine milk (14,200); Cyt, cytochrome c from horse heart (12,500); Mix, mixture of these standard proteins.

using calibrated Ultrogel AcA 34 columns yielded values of 83,000. This range indicates that PTPS does not behave exactly as a globular protein, but interacts strongly with, e.g., the AcA 34 matrix. This might be related to the possible carbohydrate content of the protein. In fact, staining of PAGE gels with Schiff's reagent indicated a band corresponding to the PTPS activity.

A question which has received considerable attention recently (14), pertains to the number of enzymes required for the conversion of H_2NTP to BH_4. Since several chemical steps are necessary (phosphate elimination, internal redox conversion, reduction of C(1') and of C(2') carbonyl functions), these events could be catalyzed by one or more proteins in addition to PTPS, and probably to sepiapterin reductase, which have been shown previously to be competent in the reaction. In our experiments, using our PTPS preparation of apparent homogeneity, and the sepiapterin reductase of the highest purity available with our procedure (3), the substrate H_2NTP was, in the presence of 10 mM DTE, Mg^{2+}, and NADPH, converted to BH_4 with substantial yield. From this we conclude, that PTPS and sepiapterin reductase are the minimal requirement for this biosynthesis. On the other hand, and in view of the rather strange dependence of BH_4 formation on sepiapterin reductase concentration, it cannot be excluded that further enzymes do play a (supplementary?) role in the process.

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