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PURIFICATION AND PROPERTIES OF THE PHOSPHATE ELIMINATING ENZYME INVOLVED IN THE BIOSYNTHESIS OF BH_4 IN MAN

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Summary: An enzyme catalyzing the elimination of triphosphate from 7,8-dihydroneopterin triphosphate in the presence of Mg^{2+} has been purified approx. 3000 fold from human liver. It has a molecular weight of approx. 63'000, a pI value of 4.4 - 4.6 and is stable at 80°C for 5 min. This enzyme catalyzes the formation of tetrahydrobiopterin in the presence of sepiapterin reductase, Mg^{2+} and NADPH. It is thus possible, that it also catalyzes the internal oxidoreduction leading to formation of the intermediate 6-pyruvoyl-tetrahydropterin, suggesting that no further enzyme is obligatory for biosynthesis of tetrahydrobiopterin.

Recent results have indicated that BH_4 [1] biosynthesis from NH_2P_3 [1] proceeds through tetrahydropterin intermediates (1-6). It is known that "Enzyme A" from Drosophila melanogaster (6,7) and "fraction A2" from chicken kidney (8) catalyze the elimination of phosphate from NH_2P_3. One product has been suggested to be 6-pyruvoyl-tetrahydropterin (2-6,9). The second is likely to be triphosphate, however, this remains to be demonstrated experimentally. In this communication we describe the purification from human liver and some properties of PEE [1], an enzyme catalyzing elimination of triphosphate from NH_2P_3.

MATERIALS AND METHODS

All pterins were purchased from Dr. B. Schircks Laboratories, Buechstrasse 17a, 8645 Jona, Switzerland. NH_2P_3 was prepared as previously described (2). Hydroxyapatite was from Bio-Rad; Ultrogel AcA 44 and the Ultropac TSK-G 3000 from LKB; Blue Sepharose CL 6B from Pharmacia; Triphosphate pentasodium from Sigma; charcoal Darco G 60 from Fluka. [α-^{32}P]GTP was from Amersham and was converted enzymatically to [α-^{32}P]NH_2P_3 in the presence of GTP

1) Abbreviations: NH_2P_3 = 7,8-dihydroneopterin triphosphate
BH_4 = 5,6,7,8-tetrahydrobiopterin
TLC = thin-layer chromatography
PEE = phosphate-eliminating enzyme

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cyclohydrolase I as described earlier (2). PEI-cellulose was from Merck and the radiochromatogram scanner from Bertold, FRG.

Purification of PEE and sepiapterin reductase. The enzyme source was human liver obtained from traffic accident victims. 70 g were homogenized in 3 volumes of 0.01 M potassium phosphate buffer, pH 6.8. After centrifugation (30 min, 12'000 x g) the supernatant was fractionated with ammonium sulfate between 30 and 60 % saturation. For desalting the supernatant was passed through a Sephadex G-25 column equilibrated in 0.01 M potassium phosphate buffer, pH 6.0. The eluate was applied to a hydroxyapatite column (2.6 x 28 cm) equilibrated with the same buffer. The enzymes were eluted with a 500 ml linear gradient of 10-300 mM potassium phosphate buffer, pH 6.0. At a flow rate of 1 ml/min fractions of 5 ml were collected. The PEE activity was eluted at 0.14 M buffer concentration and the sepiapterin reductase at 0.25 M. The fractions containing PEE or sepiapterin reductase were combined, concentrated and applied to a Ultrogel AcA 44 column (2.6 x 70 cm) equilibrated with 0.05 M Tris-acetate, pH 6.8. At a flow rate of 0.5 ml/min fractions of 5 ml were collected. PEE activity eluted in fractions 37 through 43, sepiapterin reductase in fractions 44 through 50. Further purification of the two enzymes was performed on a Ultropac TSK-G 3000 HPLC column equilibrated in 0.05 M Tris-acetate, pH 6.8. Affinity chromatography of sepiapterin reductase was carried out after Ultrogel AcA 44 on Blue Sepharose CL-6B equilibrated in 0.05 M potassium phosphate buffer, pH 6.0. The column was washed with the same buffer containing 0.1 M KCl. The sepiapterin reductase activity was eluted with 1 mM NADPH.
The protein content was measured using the Bio-Rad protein assay Kit (Bradford) and bovine gamma globulin as a standard.
Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (10) on 12.5 % acrylamide gel and in the presence of 0.1 % SDS. The protein was visualized by the silver staining method (11).

Assays for PEE and sepiapterin reductase activities. The assay for PEE contained the following reaction components (final concentration) in a total volume of 125 µl: 0.020 mM NH_2P_3, 8 mM $MgCl_2$, 1 mM NADPH, 5 mM DTE, 1.5 mU sepiapterin reductase after AcA 44 chromatography and PEE. After incubation for 1 hour at 37°C the reaction was stopped by adding 25 µl 0.2 M EDTA and the BH_4 produced was determined by HPLC and electrochemical detection (12). 1 unit of PEE was defined as the amount that catalyzes the production of 1 µmol BH_4 per min. Sepiapterin reductase activity was determined essentially as described by Sueoka and Katoh (13).

Determination of released ^{32}P. The reaction mixture for the analysis of the released ^{32}P contained in a total volume of 0.1 ml 0.085 mM [α-^{32}P] NH_2P_3, 8 mM $MgCl_2$ and 43 µl PEE, purified by AcA 44 chromatography, in 0.05 M Tris-acetate, pH 6.8. Prior to use, PEE was heated at 65°C for 1 min. Reactions were incubated at 37°C for 2 hours, followed by addition of cold ortho-, pyro- and triphosphate (as standards) and adsorption of the organic phosphates on charcoal analogously to the method of Foor and Brown (14). One part of the filtrate was counted in a liquid scintillation counter, the other part was concentrated to dryness and the residue dissolved in 50 µl of water. TLC [1]) of the released ^{32}P was carried out on PEI-cellulose (Merck). The chromatograms were developed for 15 min in 1 M LiCl in 1 M aq. acetic acid (6 cm above the origin) and afterwards, without intermediate drying in 1.5 M LiCl in 1 M aq. acetic acid until the solvent front had reached the top of the plate (17.5 cm above the origin) (15). The developed chromatogram was scanned for radioactivity. The positions of the standard phosphatases were detected with 1 % ammonium molybdate in H_2O followed by 1 % $SnCl_2$ in 10 % HCl (16). Direct analysis of the released ^{32}P was performed with TLC on cellulose (methanol : formic acid (98 %) : water, 80 : 15 : 5). 10 µl of the incubation mixture were applied.

RESULTS

Table 1 summarizes the results of a typical purification of human liver PEE. The hydroxyapatite column gave a complete separation of PEE from sepiapterin reductase. The enzyme was purified approximately 3000-fold, with a ~1.8 % recovery. By gel filtration on Ultrogel AcA 44 the molecular weight of PEE was estimated to be approx. 63'000. This molecular weight is somewhat lower than that reported for "Enzyme A" from Drosophila (MW 82'000) and for "fraction A2" from chicken kidney (MW 77'000) (7,8).

The purified PEE (main fraction of HPLC chromatography) shows one main and two minor bands on sodium dodecyl sulfate - polyacrylamide gel electrophoresis. Preparative isoelectric focussing (pH range 4-9, Servalyt T 4-9) on G-200 superfine (17) revealed an isoelectric point of 4.4-4.6. PEE is heat stable. When heated at 80°C for 5 min about 70 % of the original activity is recovered. Sepiapterin reductase is heat sensitive and was completely inactivated by this heat treatment. Thus, in the absence of sepiapterin reductase, no BH_4 was produced, although phosphate was eliminated; in spite of this we could not detect any other tetrahydropterins by HPLC.

Incubation of NH_2P_3 with Mg^{2+} and PEE after hydroxyapatite purification and subsequent oxidation with iodine under acid conditions resulted in production of pterin. Importantly, the amount of pterin produced is equivalent to the BH_4 production found in the presence of NADPH and sepiapterin reductase.

Table 1: Purification of PEE from human liver (70 g)

	Volume ml	Protein mg	Activity pmol·min^{-1}	Specific Activity pmol·min^{-1}·mg^{-1}	Recovery %
Supernatant	190	10'326	12'003	1.16	100
Ammonium sulfate (30-60 %)	33	4'896	10'802	2.21	90
Sephadex G-25	80	3'120	9'326	2.99	78
Hydroxyapatite	4.5	48.2	1'519	31.5	12.7
Ultrogel AcA 44	1.7	3.15	790	251	6.6
Ultropac TSK-G 3000	4.0	0.0714	213	2'983	1.8

Table 2: Yield of BH_4 after incubation of NH_2P_3 with 1.5 nU of PEE and 0.24 mU of sepiapterin reductase after various purification steps

	Sepiapterin reductase purified successively by			
	1.Hydroxy-apatite	2.Ultrogel AcA 44	3.Ultropac TSK-G 3000	4.Blue Sepharose CL-68
	pmol BH_4/min			
PEE purified successively by				
1.Hydroxyapatite	-	-	1.54	1.14
+Heat denaturation*	-	-	1.19	0.75
2.Ultrogel AcA 44	-	-	1.00	0.52
+Heat denaturation	-	-	0.86	0.28
3.Ultropac TSK-G 3000	0.76	0.44	0.90	0.51
+Heat denaturation	-	-	0.69	0.20

A mixture of Tris-HCl pH 7.4 (60 mM), NH_2P_3 (24 μM), $MgCl_2$ (8 mM), NADPH (1 mM), DTE (5 mM), PEE (1.5 nU) and sepiapterin reductase (0.24 mU) in 125 μl was incubated for 1 hour at 37°C. The reaction was stopped by adding 25 μl of 0.2 M EDTA.
* Heat denaturation: 5 min 80°C.
- Not measured

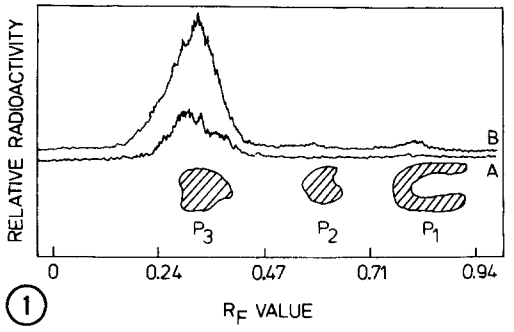
In order to obtain information whether a third enzyme is required for the production of BH_4, we incubated NH_2P_3 with constant activities of PEE (1.5 nU) and sepiapterin reductase (0.24 mU), after each purification step (Table 2). BH_4 production remained fairly constant. A large excess of sepiapterin reductase was necessary in all cases for optimal BH_4 production.

Experiments with [α-^{32}P]NH_2P_3 should confirm the hypothesis that PEE is catalyzing the elimination of phosphate. Analysis of the released ^{32}P was performed on two different TLC-systems to differentiate between ortho-, pyro- or triphosphate: Before sample application [α-^{32}P]NH_2P_3 was removed by adsorption on charcoal as described by Foor and Brown (14), because on PEI-cellulose inorganic triphosphate is not separated from residual [α-^{32}P]NH_2P_3. However, during this procedure some nonenzymatic elimination of labeled triphosphate from [α-^{32}P]NH_2P_3 was observed. PEE induced elimination, however, was at least 3 times higher. (Fig. 1) That triphosphate was eliminated was also shown by TCL on cellulose. In this case, the sample was not pretreated with charcoal, because organic phosphates remained close to

the origin and did not interfere with inorganic triphosphate. No triphosphate elimination occured in the control (incubation of [α-^{32}P]NH_2P_3 without PEE). The amount of enzymatically released ^{32}P (conversion in % from [α-^{32}P]GTP) was equal to the amount of BH_4 produced in a parallel standard incubation containing excess sepiapterin reductase.

DISCUSSION

The conversion of NH_2P_3 to BH_4 involves, as has been discussed elsewhere (4), three chemically distinct steps: Elimination of triphosphate, internal oxidoreduction and reduction of the oxo groups formed. The exact sequence of these events has not been determined, and the enzyme(s) catalyzing them have not been characterized in detail. The results described in this communication clearly establish that triphosphate is eliminated, and that this is an early event, if not the first one, in the sequence (Fig. 2) . This is in agreement with similar suggestions and proposals of others (1-6,18). From studies with labeled NADP*H (19) it is unlikely that sepiapterin reductase would catalyze the internal oxidoreduction leading to formation of 6-pyruvoyl-tetrahydro-



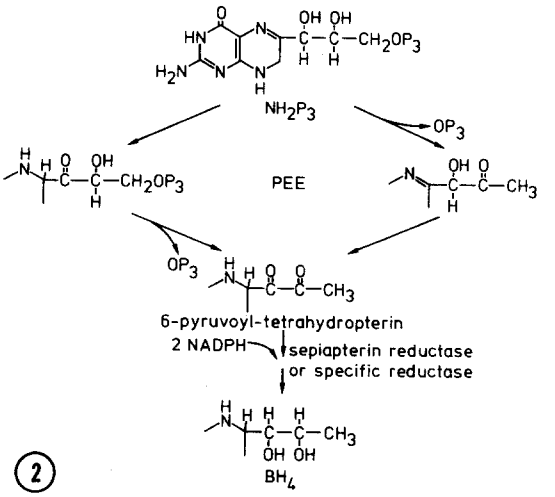


Fig. 1. Thin layer chromatogram on PEI-cellulose of products from incubation of [α-^{32}P]NH_2P_3 with PEE. For details see Materials and Methods. (A) Control: reaction mixture without PEE, (B): reaction mixture with PEE. The hatched areas represent zones visualized with ammonium molybdate and identified with standards of P_1, P_2 and P_3.

Fig. 2. Possible reaction sequence of biosynthesis of tetrahydrobiopterin.

pterin. Therefore, it follows that PEE might be involved also in this oxido-reduction step. This would be in agreement with the results of Switchenko et al. (6) obtained with Drosophila extracts.

Heat treatment of purified PEE does not alter its ability to catalyze BH_4 formation. Since sepiapterin reductase is heat labile, and since heat treated PEE alone does not catalyze BH_4 formation, it is unlikely, that the protein contaminants present in the purest fractions of PEE would represent other reductases, catalyzing the reduction of the intermediate to BH_4. On the other hand, the requirement of a large excess of sepiapterin reductase for efficient BH_4 formation, still remains a puzzle. Ours, and the results of others (6,20) cannot exclude, that still another enzyme is involved in the reduction of the two oxo groups of the proposed intermediate, sepiapterin reductase being only an unspecific surrogate for this.

Finally, it should be mentioned, that by miniaturisation of the assay, PEE activity could be measured in human liver biopsies, and most importantly, that in patients with "BH_2-synthetase deficieny" PEE activity was absent (21).

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