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Biosynthesis of tetrahydrobiopterin

Purification and characterization of 6-pyruvoyl-tetrahydropterin synthase from human liver

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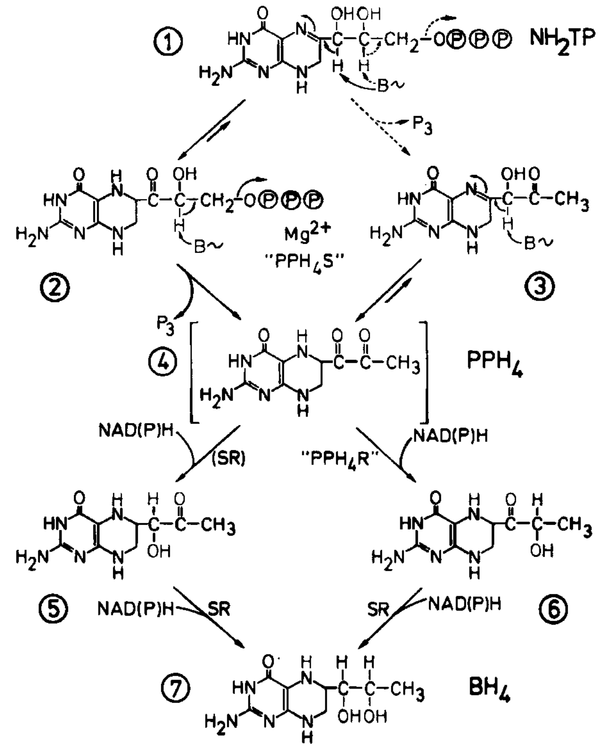
6-Pyruvoyl-tetrahydropterin synthase, which catalyzes the first step in the conversion of 7,8-dihydroneopterin triphosphate to tetrahydrobiopterin, was purified approximately 140,000-fold to apparent homogeneity from human liver. The molecular mass of the enzyme is estimated to be 83 kDa. 7,8-Dihydroneopterin triphosphate was a substrate of the enzyme in the presence of Mg^{2+}, and the pH optimum of the reaction was 7.5 in Tris HCl buffer. The K_m value for 7,8-dihydroneopterin triphosphate was 10 µM. The product of this enzymatic reaction was the presumed intermediate 6-pyruvoyl-tetrahydropterin. This latter compound was converted to tetrahydrobiopterin in the presence of NADPH and partially purified sepiapterin reductase from human liver. The conditions and the effect of *N*-acetylserotonin on this reaction, and on the formation of the intermediates 6-(1′-hydroxy-2′-oxopropyl)-tetrahydropterin and 6-(1′oxo-2′-hydroxypropyl)-tetrahydropterin have been studied.

Tetrahydrobiopterin (BH_4, *7*) (cf. Scheme 1, below) is the cofactor of aromatic amino acid hydroxylases such as phenylalanine hydroxylase (EC 1.14.16.1), tryptophan hydroxylase (EC 1.14.16.4), and tyrosine hydroxylase (EC 1.14.16.2) [1]. Therefore it plays an important role in the biosynthesis of the neurotransmitters catecholamine and indolamine [2]. Inborn errors in BH_4 biosynthesis cause atypical phenylketonuria [3–10]. From this viewpoint, it is very important to clarify the biosynthetic pathway of BH_4 in man and its modes of regulation.

It is generally accepted that the first reaction in the biosynthesis of BH_4 is catalyzed from GTP by a single enzyme, GTP cyclohydrolase I (EC 3.5.4.16), the enzymatic product being NH_2TP (*1*). This enzyme has been isolated from various sources, from *Drosophila melanogaster* [11], from *Escherichia coli* [12], and from human liver [13]. The further transforma-



Scheme 1. *Proposed pathway for the biosynthesis of tetrahydrobiopterin from dihydroneopterin triphosphate in human liver.* The pathway is shown as to proceed from NH_2TP (*1*) to BH_4 (*7*) via alternative (and equivalent) pathways and through the key intermediate 6-pyruvoyl-tetrahydropterin (PPH_4, *4*). The structure of this latter intermediate has not yet been proven directly; the compound might also exist in a hydrated or equivalent form. The two first steps in the sequence are catalyzed by a single enzyme, 'PPH_4-synthase' ('PPH_4S'). In this enzyme the same active center base (B ~) is shown to catalyze the abstraction as a proton of both the 1′-H (→), as well as the 2′-H (– – – →) of the side chain. The enzymes which catalyze the two-step reduction of PPH_4 (*4*) to BH_4 (*7*) are 'PPH_4 reductase' and sepiapterin reductase (SR), respectively. The sequence of the two steps, i.e. whether reduction occurs first at C-1′ or at C-2′, to form intermediates *5* or *6*, has not yet been determined

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Abbreviations and trivial names. BH_4, tetrahydrobiopterin, the trivial name for 2-amino-4-oxo-6-(L-*erythro*-1′,2′-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; NH_2TP, dihydroneopterin triphosphate, 2-amino-4-oxo-6-(D-*erythro*-1′,2′,3′-trihydroxypropyl)-7,8-dihydropteridine 3′-triphosphate; sepiapterin, 2-amino-4-oxo-6-lactoyl-7,8-dihydropteridine; BH_2, dihydrobiopterin, 2-amino-4-oxo-6-(L-*erythro*-1′,2′-dihydroxypropyl)-7,8-dihydropteridine; HPLC, high-performance liquid chromatography. Following a convention agreed upon at the 4th International Symposium on Biochemical and Clinical Aspects of Pteridines [see *Biochemical and Clinical Aspects of Pteridines*, vol. 4, p. 256 (1985)] the following names and abbreviations will also be used: PPH_4, 6-pyruvoyl-tetrahydropterin, 2-amino-4-oxo-6-pyruvoyl-5,6,7,8-tetrahydropteridine; PPH_4S, 6-pyruvoyl-tetrahydropterin synthase; the enzyme reducing PPH_4 to 6-lactoyl-tetrahydropterin in the presence of NADPH is thus tentatively named 6-pyruvoyl-tetrahydropterin reductase.

Enzymes. Phenylalanine hydroxylase (EC 1.14.16.1); tryptophan hydroxylase (EC 1.14.16.4); tyrosine hydroxylase (EC 1.14.16.2); GTP cyclohydrolase I (EC 3.5.4.16); sepiapterin reductase (EC 1.1.1.153); dihydrofolate reductase (EC 1.5.1.3); dihydropteridine reductase (EC 1.6.99.7).

thickness into pieces of 5-mm length, homogenization with 0.02 M potassium phosphate buffer, pH 7.0, centrifugation, and assay of the supernatant for enzyme activity. Electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate was performed as described by Laemmli [42].

Determination of molecular mass

The molecular mass of the native enzyme was estimated by gel filtration as described by Andrews [43] using Ultrogel AcA 34, Ultrogel AcA 44 and Fractogel TSK HW-50 (S) as media. Columns of 1.6 × 90 cm were employed and they were equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, containing 0.2 M KCl. A purified enzyme solution was applied to the columns. Fractions from the columns were assayed for enzyme activity. The following proteins were used as the standard markers: sweet potato β-amylase (M_r 200000), yeast alcohol dehydrogenase (150000), pig heart diaphorase (≈ 100000), bovine serum albumin (66000), ovalbumin (45000), bovine erythrocyte carbonic anhydrase (29000), equine skeletal muscle myoglobin (17800), and horse heart cytochrome *c* (12400). Elution of the marker proteins was monitored by the absorbance at 280 nm.

RESULTS

Purification of PPH₄S

PPH₄S was purified from 500-g aliquots of human liver. All procedures were carried out at 4 °C unless otherwise specified.

Step 1. Crude extract. Human liver (250 g) was thawed and cut into small pieces and homogenized in a Virtis-45 homogenizer for 3 min with 2 vol. 0.01 M potassium phosphate buffer, pH 7.0 at half of full speed. The homogenate was blended with another portion of 2 vol. of the same buffer for an additional 3 min at two-thirds full speed and then it was centrifuged at 27000 × *g* for 60 min. The resulting supernatant was filtered through cheesecloth to remove fat.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant to 35% saturation, the mixture was stirred for 40 min, the precipitate was removed by centrifugation at 11 000 × *g* for 60 min, and the supernatant was filtered again through cheesecloth to remove the last residues of fat. Additional solid ammonium sulfate was added to the resulting supernatant to 55% saturation. The mixture was stirred for 40 min and the precipitate was collected by centrifugation at 11000 × *g* for 60 min. The precipitated protein was dissolved with 80 ml of 0.01 M potassium phosphate buffer, pH 6.0, and dialyzed overnight against 10 l of the same buffer. The dialyzed solution was stored at − 20 °C until used. The ammonium sulfate fraction was also prepared from another batch of 250 g human liver and the fractions from two batches were combined.

Step 3. Column chromatography on hydroxyapatite. The ammonium sulfate fractions obtained from two batches (i.e. starting from a total of 500 g liver) were thawed and combined, and the solution was centrifuged at 27000 × *g* for 60 min in order to remove precipitated protein. The supernatant solution was applied to a hydroxyapatite column (5 × 45 cm) that had been equilibrated with 0.01 M potassium phosphate buffer, pH 6.0. After the column was washed with 300 ml of the buffer, the adsorbed proteins were eluted by a linear gradient with 1000 ml of 0.01 M potassium phosphate buffer, pH 6.0, in the mixing chamber and 1000 ml of 0.2 M potassium phosphate buffer, pH 6.0, in the reservoir. The flow rate was maintained at 80 ml/h and 20-ml fractions were collected. Sepiapterin reductase was retained on hydroxyapatite under the above condition; it was therefore also eluted with 0.3 M potassium phosphate buffer, pH 6.0, after collection of PPH₄S. The elution profile is shown in Fig. 1.

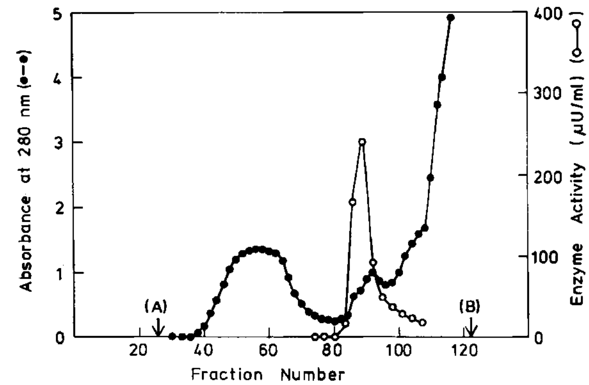

Fig. 1. *Elution profile of PPH₄S from hydroxyapatite column.* (●) Protein concentration measured by absorbance at 280 nm; (○) enzyme activity, measured by use of 50 μl of protein solution under the standard assay conditions. Arrow (A) indicates the starting point of gradient of phosphate buffer and arrow (B) indicates the end of gradient

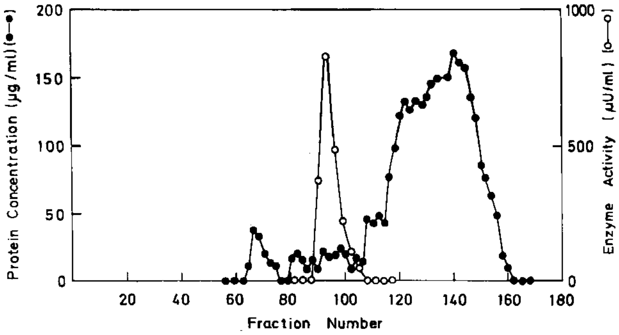

Fig. 2. *Elution profile of PPH₄S from Ultrogel AcA 44 column.* (●) Protein concentration measured by Bio-Rad protein assay kit; (○) enzyme activity, measured by use of 30 μl of protein solution under the standard assay conditions

Step 4. Heat treatment. Fractions from the hydroxyapatite column containing PPH₄S activity were combined and concentrated with an Amicon PM-10 ultrafilter to about 50 ml. Since PPH₄S is heat-stable [15, 26, 33], this solution was heated for 5 min at 80 °C with gentle shaking in a water bath. The heat-treated suspension was cooled in an ice bath for 3 min, and centrifuged at 17000 × *g* for 30 min to remove denatured proteins.

Step 5. Gel filtration on Ultrogel AcA 44. The supernatant from the previous step was applied to an Ultrogel AcA 44 column (5 × 91 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.0, containing 0.2 M KCl. The column was developed with the same buffer and 10-ml fractions were collected at a flow rate of 50 ml/h. The elution profile is shown in Fig. 2. Active fractions were collected, combined, and concentrated by Amicon PM-10 ultrafiltration to about 8 ml and dialyzed overnight against 5 l 0.02 M potassium phosphate buffer, pH 7.0.

Step 6. Column chromatography on DEAE-Fractogel 650 S. The solution obtained from the previous step was applied to a DEAE-Fractogel 650 S column (2.6 × 34 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.0. After washing with 80 ml of the same buffer, proteins were eluted with a linear gradient of 400 ml 0.02 M potassium phosphate buffer, pH 7.0, in the mixing chamber and 400 ml of the same buffer containing 0.4 M KCl in the reservoir. Each 10-ml fraction was collected at a flow rate of 40 ml/h. The elution profile is shown in Fig. 3. Active fractions were collected and combined. The combined solution was concentrated by Amicon PM-10 ultrafiltration into a small volume and dialyzed overnight against 5 l 0.01 M potassium phosphate buffer, pH 7.0.

Table 1 presents a summary of a typical purification of PPH_4S from 500 g human liver. By this procedure the enzyme was purified approximately 140000-fold with an overall recovery of about 60%. The purification of PPH_4S by this procedure has been repeated four times and the protein elution profiles from the various columns are completely reproducible; however, the purification factor and the overall recovery varied in the ranges of 40000–150000 and 33–61%, respectively, depending on the batches of liver used.

The enzyme was not homogeneous at this level of purification. When the purified enzyme was subjected to electrophoresis on polyacrylamide gel, one major protein band and two minor bands were observed (Fig. 4A). When proteins were extracted from polyacrylamide gel after electrophoresis and subjected to enzyme assay, PPH_4S activity corresponded very well to the main protein band. Proteins corresponding to other minor bands did not affect BH_4 formation. In order to obtain PPH_4S of apparent homogeneity, PPH_4S from the DEAE-Fractogel purification step was subjected to preparative polyacrylamide gel electrophoresis using a 1-mm-thick slab gel. Electrophoresis of the material obtained was homogeneous (Fig. 4B). This homogeneous preparation of the enzyme was concentrated again by Amicon PM-10 ultrafiltration (≈ 0.2 mg/150 µl); it can be stored at −70 °C for several months without appreciable loss of activity. The results to be presented in the following sections of this paper were obtained with this homogeneous enzyme preparation.

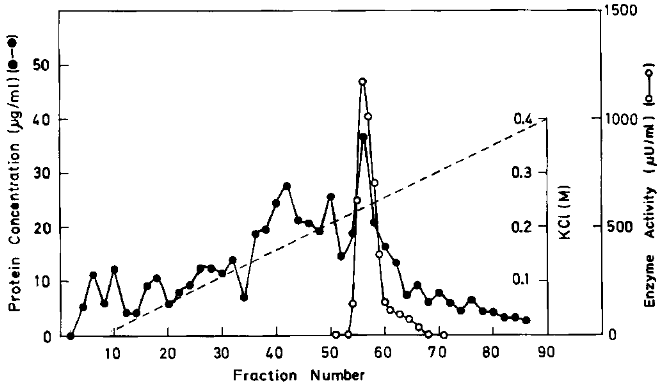

Fig. 3. *Elution profile of PPH_4S from DEAE-Fractogel 650 S column.* (●) Protein concentration measured by Bio-Rad protein assay kit; (○) enzyme activity, measured by use of 10 µl of protein solution under the standard assay conditions; (– – – –) KCl concentration

Properties of the enzyme

The enzymatic synthesis of BH_4 (*7*) from NH_2TP (*1*) (cf. Scheme 1) was found to be linear with time up to at least

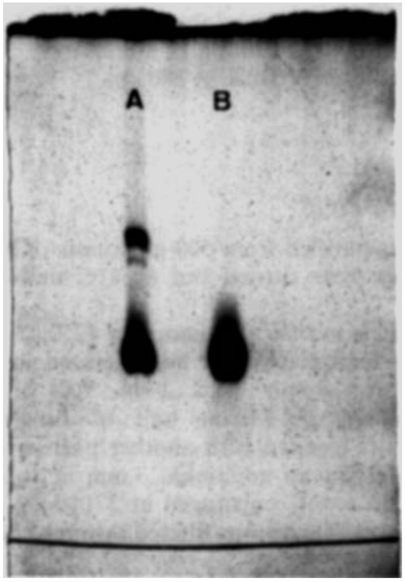

Fig. 4. *Electrophoresis of PPH_4S on polyacrylamide gel.* Electrophoresis was performed on 7% polyacrylamide gel as described under Experimental Procedures. (A) Enzyme preparation after DEAE-Fractogel 650 S step; (B) enzyme preparation after preparative electrophoresis. Approximately 10 µg protein was loaded. The proteins were stained with Bio-Rad silver-stain kit

Table 1. *Summary of purification of PPH_4S*

Enzyme preparation	Volume	Total activity	Total protein	Specific activity	Recovery	Purification
	ml	mU	mg	mU/mg	%	-fold
Crude extract	1890	50.0	72500	0.0007	100	1.0
Ammonium sulfate, 35–55% fraction	280	45.5	11600	0.004	91	5.7
Hydroxyapatite eluate	400	43.5	230	0.19	87	269
Heat-treated hydroxyapatite eluate	40	42.0	49	0.86	84	1224
Ultrogel AcA 44 eluate	160	36.0	1.7	21.2	72	30252
DEAE-Fractogel 650 S eluate	70	30.0	0.3	100.0	60	143000

One unit of activity is defined as the amount of enzyme which catalyzes the production of 1 µmole BH_4/min from NH_2TP at 37 °C under standard assay conditions. The activity of the crude extract was measured in the absence of dithioerythritol with an enzyme preparation which was heat-treated at 80 °C for 5 min; without heating, activity decreased to 35%, and about 55% of NH_2TP was converted to dihydroneopterin by phosphatases during the incubation; in the presence of 10 mM dithioerythritol activity decreased to 50–70%. The activity of the ammonium sulfate fraction was measured with enzyme heat-treated at 80 °C for 5 min; without heating, activity decreased to 45%, and about 60% of NH_2TP was converted to dihydroneopterin by phosphatases

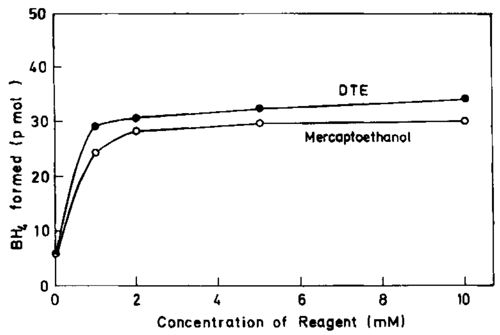

Fig. 5. *Effect of reducing reagent at various concentrations on BH_4 formation*. The standard reaction mixtures, containing various amounts of dithioerythritol or mercaptoethanol, were incubated for 60 min at 37°C with 9 μU PPH_4S. Aliquots of 10 μl from the incubated mixtures were injected into the HPLC system and the amount of BH_4 produced was measured. (●) Amount of BH_4 produced in the presence of dithioerythritol (DTE); (○) amount of BH_4 produced in the presence of mercaptoethanol

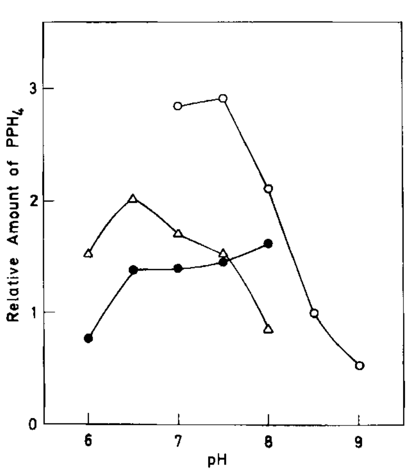

Fig. 6. *Effect of pH and of buffer on the activity of PPH_4S*. The reaction mixture contained the following components: 17 μU PPH_4S, 8 mM $MgCl_2$, 10 mM dithioerythritol, 32 μM NH_2TP, 100 mM buffer at various pH values in the final volume of 125 μl. (○) Tris HCl buffer; (●) potassium phosphate buffer; (△) imidazole HCl buffer. The reaction mixtures were packed into the vials for automatic sampler of HPLC with N_2 and incubated for 30 min at 37°C in the dark. Each 10 μl aliquot of the incubated mixture was injected into the HPLC system and the peak area of PPH_4 (Fig. 8 A) was measured

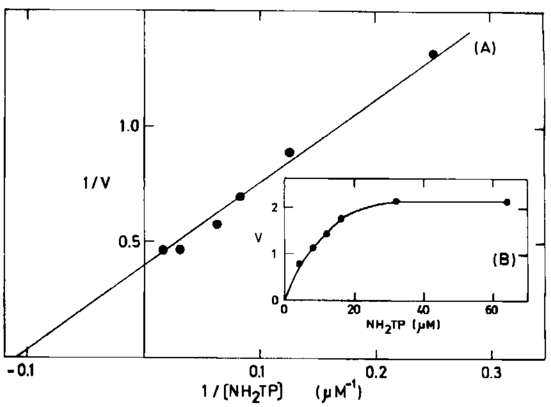

Fig. 7. *Effect of NH_2TP concentration on the rate of the PPH_4 production*. The reaction mixture contained the following components: 14 μU PPH_4S, 100 mM Tris HCl buffer, pH 7.4, 8 mM $MgCl_2$, 10 mM dithioerythritol and the indicated amount of NH_2TP in a final volume of 125 μl. The reaction mixtures were flushed with N_2 gas and incubated for 30 min at 37°C in the dark, then 10 μl of the reaction mixture was directly injected into the HPLC analysis system without stopping the reaction. The relative velocity was calculated from the area of PPH_4. (A) Double-reciprocal plot; (B) initial rates

60 min of incubation when an excess of sepiapterin reductase was used in the standard reaction mixture. Sepiapterin reductase, NADPH, and Mg^{2+}, as well as PPH_4S, were essential for BH_4 (*7*) production from NH_2TP (*1*); if one of these components was omitted from the standard reaction mixture, BH_4 formation did not occur at all. Using pure PPH_4S, BH_4 formation was increased in the presence of dithioerythritol as shown in Fig. 5. However, when a rather crude preparation of the enzyme was used for this experiment, enhancement of BH_4 formation was not observed; on the contrary, the activity of PPH_4S in crude extracts was decreased by 30–50% by the addition of 10 mM dithioerythritol. For this reason PPH_4S activity was measured in the absence of dithioerythritol during the early step of purification. A 20-min pre-incubation of PPH_4S with 1 mM dithioerythritol, followed by incubation in the presence of 2% of this concentration, i.e. 0.02 mM, led to an increased activity comparable to that shown in Fig. 5. The effect of dithioerythritol on pure enzyme probably resides in the reduction of (essential) disulfide bridges of PPH_4S; the effect observed with crude extracts, however, is as yet unexplained. The enzymatic production of PPH_4 (*4*) from NH_2TP (*1*) in the presence of Mg^{2+} and PPH_4S was linear with time up to at least 30 min of incubation under anaerobic conditions. In the absence of Mg^{2+} no PPH_4 (*4*) was produced. The activity optimum of the enzyme was pH 7.5 in Tris/HCl buffer as shown in Fig. 6. It is apparent from the dependencies shown in Fig. 6, that the nature of the buffer as well as its ionization state play an important role in catalysis. The effects observed might also originate from interactions of buffers such as Tris or imidazole with Mg^{2+}. PPH_4 formation from NH_2TP exhibited typical Michaelis-Menten kinetics. The K_m value for NH_2TP was estimated to be 10 μM from a double-reciprocal plot [44] as shown in Fig. 7.

The molecular mass of the enzyme was estimated to be about 83 kDa from the elution volume using three different gel filtration columns, AcA 34, AcA 44, and Fractogel TSK HW-50 (S), which were calibrated with standard proteins. The results obtained by use of the three different media gave approximately the same value of the molecular mass of PPH_4S. When the purified enzyme was subjected to electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate, a single band with an estimated molecular mass of about 19 kDa [34] was obtained. These results suggest that PPH_4S consists of four subunits of identical molecular mass.

Studies on the conversion of NH_2TP and on the pathway of BH_4 synthesis

When NH_2TP (*1*) was incubated with PPH_4S in the presence of Mg^{2+} under anaerobic conditions, it was converted to a compound which is detectable electrochemically under conditions which are specific for tetrahydropterins [36] as shown in Fig. 8 A. This compound has been proposed to be PPH_4 (*4*), which is supposed to be unstable; its structure is shown in Scheme 1 [22, 24–26]. NADPH was not required for this transformation, confirming previous results [24].

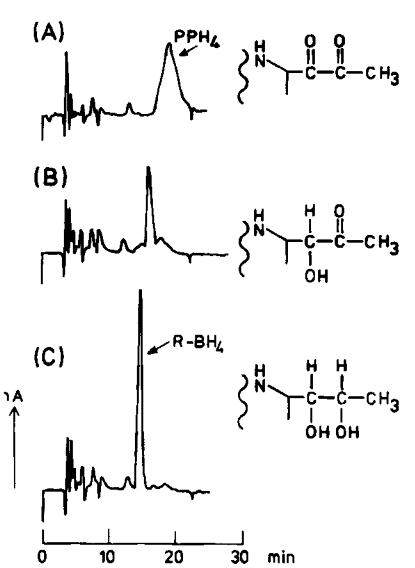

Fig. 8. *HPLC chromatograms of tetrahydropterins with electrochemical detection.* (A) The reaction mixture containing 32 µM NH_2TP, 100 mM Tris HCl buffer (pH 7.4), 10 mM dithioerythritol, 8 mM $MgCl_2$ and 17 µU PPH_4S in a final volume of 125 µl was flushed with N_2 gas and then incubated for 30 min at 37 °C in the dark. The volume injected for analysis was 10 µl. (B) The same reaction mixture as in experiment A was incubated for 30 min at 37 °C, and then 20 mM EDTA, 0.1 mM *N*-acetylserotonin, 2 mU sepiapterin reductase and 0.5 mM NADPH (final concentration) were added and further incubated for 7 min at 37 °C. The final volume of the reaction mixture was 250 µl and 20 µl was injected into the HPLC system. (C) The same conditions as in experiment B, but the second incubation was done without *N*-acetylserotonin

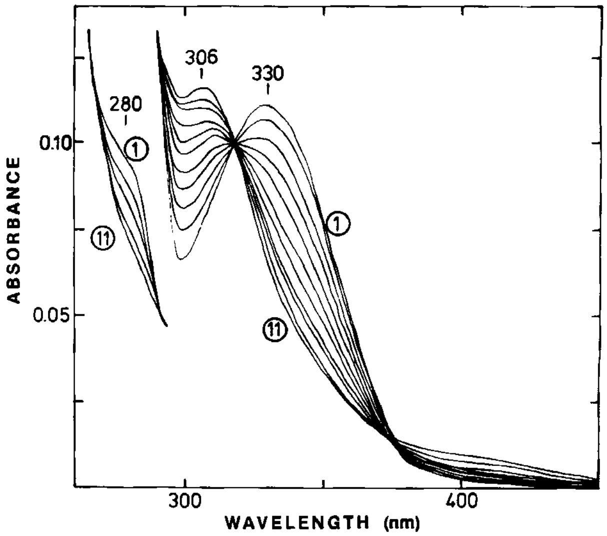

Fig. 9. *Effect of PPH_4 synthase on the absorption spectrum of NH_2TP.* A volume of 1.0 ml 0.1 M Tris HCl buffer pH 7.4, containing 8 mM $MgCl_2$, 10mM dithioerythritol and 16 µM NH_2TP was made anaerobic by repeated cycles of N_2-flushing and evacuations at 0 °C. 0.5 mU PPH_4S in 50 µl of the same buffer were then added anaerobically from a side arm at 25 °C and spectrum 1 was recorded immediately. Spectra 2–11 were recorded after 3.5, 6, 8.5, 11, 15, 20, 25, 30, 40 and 60 min, respectively. For clarity only selected spectra are shown below 300 nm

It has been proposed [22, 31] that the catalytic action of PPH_4S involves the conversion of a dihydropterin chromophore, which is characterized by an absorption maximum at 330 nm in the near-ultraviolet, to a tetrahydrochromophore. The latter does not exhibit prominent absorption in this region, thus allowing a spectroscopic monitoring of the process. Switchenko et al. [22] have indeed shown, that partially purified preparations from *D. melanogaster* induce changes in the spectrum of NH_2TP which are consistent with the proposed change of the redox status. Essentially the same conversion was obtained using our pure PPH_4S as shown in Fig. 9.

Clearly, the conversions shown are compatible with the disappearance of the dihydropteridine chromophore. Although the general pattern of the conversion is similar to that observed by Switchenko et al. [22], some differences are also apparent. In our case, a compound with a pronounced maximum just above 300 nm is formed after 1 h of incubation. This is consistent with formation of a tetrahydropterin chromophore since the latter has a λ_{max} around 300 nm (e.g. BH_4, $\lambda_{max} = 297$ nm [45]). The minor difference in λ_{max} can arise from the different side chain at position 6 and also from the presence of enzyme in our incubation mixture. During the first 50% of the conversion there appears to be only marginal absorption in the 380–450 nm region. This again contrasts with the formation of a maximum at 400 nm observed with the *Drosophila* system [22]. It is not clear that a structure such as that of PPH_4 (*4*) should have such an absorbance which is typical of 1′-oxo-dihydropterins such as sepiapterin, since the triaminopyrimidine chromophore is electronically separated from the side-chain carbonyl groups. The appearance of this absorbance is maximal in the last stage of the incubation (Fig. 9). It is thus probable that it originates from decay products of PPH_4. Analysis of the incubation mixture obtained after 60 min with HPLC indicates complete disappearance of NH_2TP and formation of PPH_4.

Investigations of the conversion of PPH_4 (*4*) to BH_4 (*7*) are hampered by the lability of the former. They were thus carried out with material generated by incubation of NH_2TP with PPH_4 without isolation. This was done by addition of EDTA to the incubation mixture, which stops the activity of PPH_4S by complexation of Mg^{2+}.

Several experiments were carried out with PPH_4 generated by this method. Subsequent addition of sepiapterin reductase and NADPH to this reaction mixture (cf. legend to Fig. 8 for details), leads to formation of BH_4 (*7*) (Fig. 8C). Qualitatively the same result was obtained using PPH_4 (*4*) which was purified by HPLC, as will be detailed elsewhere (Leimbacher, W. and Curtius, H.-Ch., unpublished work). The same secondary incubation of PPH_4 with sepiapterin reductase and NADPH was also carried out in the presence of *N*-acetylserotonin, a potent inhibitor of sepiapterin reductase [46]. In this case (Fig. 8B), PPH_4 (*4*) was converted to a compound having a retention time coinciding with that of 6-(1′-hydroxy-2′-oxopropyl)-5,6,7,8-tetrahydropterin (*5*) [28]. This contrasts with experiments in which PPH_4 (*4*) was incubated with crude homogenate of human liver (purification step 2) instead of partially purified sepiapterin reductase. In this case the reaction in the presence of *N*-acetylserotonin gave a different compound, the retention time of which coincided with that of 6-(L-lactoyl)-5,6,7,8-tetrahydropterin (*1,6* 'dihydro-sepiapterin'). When *N*-acetylserotonin was omitted, BH_4 (*7*) formation was observed as usual.

The sepiapterin reductase used for the conversions of PPH_4 was obtained after Ultrogel AcA 44 filtration; it was not homogeneous according to polyacrylamide gel electrophoresis. Attempts to purify sepiapterin reductase further by use of DEAE-Sephadex A-50 (pH 7.0), did not yield a homogeneous protein; however, when this sepiapterin reductase preparation was used for the incubation of PPH_4 (*4*), the amount of BH_4 (*7*) produced was strongly decreased although not suppressed, in spite of using the same amount of sepiapterin reductase activity determined by measuring the reduction rate of sepiapterin [39]. This indicates the presence of a further enzyme, which is not essential for production of BH_4 (*7*), but which clearly plays a catalytic role. Indeed, as will be detailed later (Takikawa, S., Heintel, D., Curtius, H.-Ch., unpublished work) further purification of fractions eluting from the hydroxyapatite column has led to isolation of an enzyme which has no sepiapterin reductase activity and is not inhibited by *N*-acetylserotonin. Incubation of PPH_4 (*4*) with this enzyme in the presence of NADPH leads to formation of 6-lactoyl-5,6,7,8-tetrahydropterin (*6*), while no further product was observed (cf. Scheme 1). A further factor affecting the yield of BH_4 (*7*) was the presence of considerable amounts of dihydropteridine reductase activity in preparations of sepiapterin reductase obtained from the DEAE-Sephadex A-50 column. Dihydropteridine reductase has been reported to be necessary to optimize formation of BH_4 in *in vitro* systems [30]. This indicates, that during these incubations partial oxidation of PPH_4 or of subsequent intermediates to the corresponding quinonoid dihydropterin forms occurs, and that the latter can be reconverted to the tetrahydro level by dihydropteridine reductase. Therefore, for general assays in which formation of BH_4 (*7*) is monitored, the use of partially purified sepiapterin reductase at the Ultrogel AcA 44 purification level (which contained a substantial amount of dihydropteridine reductase activity) proved to be optimal.

DISCUSSION

The results presented in this paper indicate that PPH_4S is a single protein which catalyzes two reactions, that is, elimination of phosphate from NH_2TP (*1*) [33] and an internal oxidoreduction leading to PPH_4 (*4*) (Scheme 1). The enzyme consists of four identical subunits of 19 kDa each, the molecular mass of tetrameric PPH_4S being 83 kDa. This value is higher than the 63 kDa reported in earlier papers [10, 28, 33]. However, we think that the higher value is more accurate, because it was obtained by analysis of the homogeneous protein and was measured by using three different media for gel filtration. PPH_4S was clearly eluted from these three different columns earlier than bovine serum albumin, which has a molecular mass of 66 kDa. The value of 83 kDa also agrees well with the value of 82 kDa reported for the same enzyme from *D. melanogaster* [14].

Total activity, purification factor and recovery (presented in Table 1) sometimes varied depending on the different batches of human liver used. In addition to the origin of the liver, the quality of the enzymatically prepared NH_2TP, which was used for the assays, was also found to affect formation of BH_4. Traces of dihydropteridine reductase, which are contained in the sepiapterin reductase used for the assays and which were found to increase the yield of BH_4, appear to be of practical importance. The role of this enzyme is assumed to consist in the reduction of pteridines which have been oxidized non-enzymatically to the quinonoid dihydro form by O_2 during the assay, to the tetrahydro level [1, 30]. On the other hand, the finding that high concentrations of dithioerythritol lower BH_4 yields when crude liver extracts are used, cannot yet be explained.

With respect to the mechanisms of the conversion of NH_2TP (*1*) to PPH_4 (*4*), it appears clear that the homogeneous enzyme can catalyze the two necessary steps [47] with equal efficacy since the conversion appears to proceed isosbestically (Fig. 9) [22] and since intermediates such as *1*, *2* or *3* (Scheme 1) have never been observed by us or by others. The experimental determination of the sequence of these two steps, i.e. elimination of triphosphate [33] and the internal oxidoreduction, might prove very difficult to achieve. It is conceivable that the same enzyme base which is assumed to induce triphosphate elimination by abstracting the 2′-hydrogen as a proton might be positioned to remove also the 1′-H (as a proton) and thus to catalyze also the internal redox shuttle (Scheme 1). The assumed intermediates *2* or *3* might thus not leave the enzyme active center during catalysis, resulting in a direct conversion of NH_2TP (*1*) to PPH_4 (*4*). From a chemical point of view [47], a prediction is difficult. Elimination of phosphate from *1* will yield the dihydropterin *3* in which the 1′-hydrogen should be considerably more acidic. The same, however, holds for *2*, in which the 2′-hydrogen should also be more acidic, as compared to 2′-H in *1*. In any event, from these considerations it is reasonable to assume that the ‘second step’ in the conversion of *1* to *4* might be faster than the first one. This would agree with the observation of no intermediates mentioned above.

In contrast to this, for the conversion of PPH_4 (*4*) to BH_4 (*7*) a clear-cut picture begins to emerge. Our results are in good agreement in particular with those of Masada et al. [30], which were obtained with homogeneous sepiapterin reductase and which showed that this single enzyme is competent in the conversion of PPH_4 (*4*) to BH_4 (*7*), although at a slow rate. From our results it appears that a further enzyme is present in human liver, which converts PPH_4 (*4*) to 6-lactoyl-tetrahydropterin (*6*) (Scheme 1) in the presence of NADPH, but apparently not further to BH_4 (*7*). This enzyme might be tentatively named 6-pyruvoyl-tetrahydropterin-2′-reductase (‘PPH_4R’) (Scheme 1). Sepiapterin reductase catalyzes the conversion of *5* to BH_4 (*7*), as has been shown using purified *5*, or by demonstration of dehydrogenation of BH_4 (*7*) to *5* in the presence of $NADP^+$ [28, 48]. On the other hand, sepiapterin reductase also efficiently reduces *6* to BH_4 (*7*) [21, 22, 25, 49]. The elucidation of details concerning the conversion of PPH_4 (*4*) to BH_4 (*7*) will have to await the purification of the presumed PPH_4 reductase to homogeneity, the study of its reaction kinetics with isolated *4*, *5* and *6*, and the comparison of these properties with those of pure sepiapterin reductase in the presence and absence of *N*-acetylserotonin. It thus appears conceivable that both pathways (i.e. lower right-hand side proceeding over intermediate *6*, or lower left-hand side over *5*, Scheme 1) might be viable depending on the specific conditions and also on the properties of these two enzymes which might differ depending on the sources. The effects on *N*-acetylserotonin on the conversions induced by sepiapterin reductase are not yet clear, although they suggest that reduction of 1′- and 2′-carbonyl groups are inhibited to different degrees.

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