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Purification and Characterization of a Carbonyl Reductase from Human Liver, which is Competent in the Reduction of 6-Pyruvoyl-Tetrahydropterin*

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Summary

An enzyme which reduces 6-pyruvoyl-tetrahydropterin has been purified to apparent homogeneity from human liver. It consists of a single polypeptide chain with a molecular weight of 35 kDa, has an isoelectric point of 5.9 ± 0.1 and contains no glycosyl residues. The pure enzyme has a specific activity of 450 mU/mg protein at pH 7.0 in 10 mM potassium phosphate buffer. It converts 6-pyruvoyl-tetrahydropterin to 6-lactoyl-tetrahydropterin by transfer of the *pro* 4R-hydrogen of NADPH to form the side chain -OH at position C(2′) of the substrate. K_m values are 1.8 µM for 6-pyruvoyl-tetrahydropterin and 5.5 µM for NADPH. Polyclonal antibodies raised against the purified enzyme recognize 6-pyruvoyl-tetrahydropterin reductase in Western blot and ELISA but do not cross-react with human sepiapterin reductase. The enzyme appears to be identical with aldose reductase.

Introduction

Tetrahydrobiopterin (BH_4) is the natural cofactor of the monooxygenases which hydroxylate phenylalanine, tyrosine and tryptophan to produce the precursors of the catecholamine and indoleamine neurotransmitters (1). Further oxidation reactions involving ether lipid oxidation (2), prolin hydroxylation (3), and mitochondrial electron transport (4) have also been discussed to require a tetrahydropterin as a cofactor; however, the only established physiological role of BH_4 is as cofactor for the aromatic amino acid hydroxylation. So far three enzymatic defects leading to a cofactor deficiency have been described to cause atypical phenylketonuria (5). In addition, neurological disorders such as Alzheimer's disease, Parkinson's disease, dystonia and others have been associated with abnormal BH_4 metabolism (6–9). For identification of predisposed individuals as well as appropriate treatment of patients with BH_4 deficiencies, it is therefore crucial to have a proper understanding of the biosynthesis of tetrahydropterins and of its regulation. The basic reactions and intermediates occurring during biosynthesis have been elucidated (10–16). There is general agreement that three enzymes, namely GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase (PPH_4 synthase) and sepiapterin reductase, are required and are sufficient for the conversion of GTP to BH_4 (17–19).

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Enzymes: Catalase (EC 1.11.1.6); glucose oxidase (EC 1.1.3.4); GTP cyclohydrolase I (EC 3.5.4.16); 6-pyruvoyl tetrahydropterin reductase/aldose reductase (alditol $NADP^+$ 1-oxidoreductase, EC 1.1.1.21); 6-pyruvoyl tetrahydropterin synthase; sepiapterin reductase (EC 1.1.1.153)

Abbreviations: BH_4, 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin or tetrahydrobiopterin; NH_2TP, 7,8-dihydroneopterin triphosphate; PPH_4, 6-pyruvoyl-5,6,7,8-tetrahydropterin; LPH_4, 6-lactoyl-5,6,7,8-tetrahydropterin; PPH_4 synthase (PPH_4S), 6-pyruvoyl-tetrahydropterin synthase; PPH_4 reductase (PPH_4R), 6-pyruvoyl-tetrahydropterin reductase.

However, in human as well as in various animal tissues another reductase has been observed. Due to its ability to reduce the key intermediate, 6-pyruvoyl-tetrahydropterin (PPH_4), to 6-lactoyl-tetrahydropterin (LPH_4) the enzyme was originally named 6-pyruvoyl-tetrahydropterin reductase (PPH_4 reductase). The biological relevance of this activity, apart from that for sepiapterin reductase, has since been discussed (10–12, 14, 20). Investigations on the relative significance of the two possible pathways of PPH_4 reduction were particularly impaired by the lack of standardized methods for the assessment of the enzyme activity of both sepiapterin reductase and PPH_4 reductase, leading to contradictory results in different laboratories (18, 19).

In 1985 Sueoka and Katoh demonstrated that rat sepiapterin reductase catalyzes the reduction of carbonyl functions of a variety of non-pteridine aromatic and aliphatic compounds (21). In addition, it was shown that the tissue distribution of sepiapterin reductase does not correlate with the rate of BH_4 biosynthesis in the respective organs (19). These findings suggest that sepiapterin reductase might have other functions *in vivo*, in addition to the established role in the BH_4 biosynthesis (19, 22). Recently it has further been shown that the distribution of PPH_4 reductase activity also does not correlate with the BH_4 content in different rat brain regions (23).

We previously reported that both sepiapterin reductase and PPH_4 reductase in human liver extracts share an affinity for PPH_4 as a substrate (24). In an attempt to better understand the pathways of the BH_4 biosynthesis we purified PPH_4 reductase from human liver to apparent homogeneity and characterized some of its salient properties. In addition we obtained polyclonal antiserum against this enzyme, and used it as a tool for the study of the role of PPH_4 reductase in the biosynthesis of BH_4. A preliminary account of this work has been presented (20). In a most recent communication we have shown, that PPH_4 reductase is biochemically and immunologically indistinguishable from aldose reductase from human brain (25). Similar conclusions were reached by Milstien and Kaufman using rat brain enzymes (26).

Material and Methods

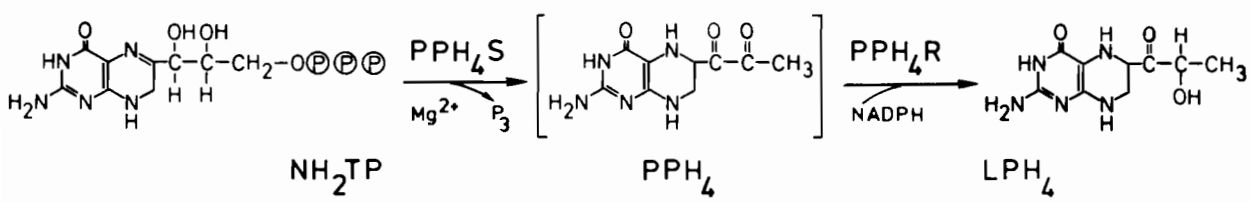
Human livers were obtained from traffic accident victims within 2 h *post mortem*. The sliced tissue was immediately frozen on dry ice and stored at −70 °C. BH_4 and sepiapterin were purchased from Dr. B. Schircks Laboratories (Buechstraße 17a, 8645 Jona, Switzerland). 7,8-Dihydroneopterin triphosphate (NH_2TP) was synthesized and purified in our laboratory, using the method of Blau (27). LPH_4 and 6-(1′-hydroxy-2′-oxo)-propyl-tetrahydropterin were prepared following the procedures described by Katoh (28) and Heintel (29). PPH_4 synthase from salmon liver and sepiapterin reductase from human liver were purified as described by Hasler (30) and Takikawa (14), respectively.

(4S)-[4-^{3}H]NADPH and (4R)-[4-^{3}H]NADPH were prepared by A. Schieber according to the method of Wermuth (31). Catalase suspension (beef liver), glucose oxidase (*Aspergillus niger*), and pyridine nucleotides were purchased from Boehringer Mannheim. Molecular weight standards and Freund's adjuvant were obtained from Sigma.

Equipment and standard reagents for Western blot procedures were products of Bio-Rad. All other chemicals were of commercially available analytical grade.

PPH_4 reductase activity assays

These were carried out by HPLC with electrochemical detection of formed LPH_4. The substrate (PPH_4) was generated *in situ* from NH_2TP using PPH_4 synthase (*cf.* Scheme 1) and its concentration changes were monitored on the HPLC profiles. One enzyme unit (U) is defined as the amount of protein catalyzing the production of 1 µmol LPH_4(min from PPH_4 at 37 °C. Unless otherwise specified, the enzyme activity was assayed in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.25 mM dithioerythritol, 8 mM $MgCl_2$, 20 µM NH_2TP, 4 mU PPH_4 synthase (from salmon liver (30)), 10 mM glucose, 12 U glucose oxidase, at least 350 U catalase, 2 mM NADPH and appropriate amounts of PPH_4 reductase, in a total volume of 100 µL. The mixtures were routinely flushed with N_2, then sealed and incubated for 8 min at 37 °C in vials suit-



Scheme 1. System used for the *in situ* production of PPH_4 and assay of PPH_4 reductase

able for automatic HPLC sampling. Reactions were terminated by adding 10 µL of a 0.5 M NaF solution containing 0.1 M dithioerythritol.

For determination of the K_m values of PPH_4 and NADPH, the PPH_4 synthase activity in the standard assay mixture was increased to 10 mU. This yields a substrate concentration which was estimated to be $\approx$ 70% of the theoretically expected value. The concentration of NH_2TP was determined by its absorption at 330 nm using the molar absorption coefficient of dihydroneopterin (6.2 mM^{-1} cm^{-1} at pH 6.8 (32)).

BH_4 was used as a stable internal standard to quantitate LPH_4. The concentrations of BH_4 standard solutions were determined using $\varepsilon_{266\,nm} = 16$ mM^{-1} cm^{-1} in 0.1 M HCl (33).

Substrate specificity

The reduction of phenylpropanedione, benzyl, glyoxal, diacetyl, and PPH_4 as a control was assayed by recording the decrease in absorbance of NADPH at 340 nm in a mixture containing 0.1 M Tris/HCl buffer, pH 7.4, 100 µM NADPH, 16 µM substrate and $\approx$ 25 PPH_4 reductase, in a total volume of 1 ml. Activities of PPH_4 reductase using sepiapterin (50 µM) or LPH_4 (25 µM) as substrate were measured by reversed-phase HPLC (fluorimetric detection) of biopterin subsequent to acid oxidation with MnO_2. 6-(1′-Hydroxy-2′-oxo)-propyl-tetrahydropterin (4 µM) conversion was analyzed by electrochemical detection of BH_4 following HPLC. Controls with sepiapterin reductase and without either reductase were routinely included.

Catalytic properties of PPH_4 reductase

For these studies the conditions were: 0.1 M Tris/HCl buffer, pH 7.4, 0.5 mM dithioerythritol, 8 mM $MgCl_2$, 50 µM NH_2TP, 2 mU PPH_4 synthase, 1 mM NADPH and about 40 µU PPH_4 reductase, in a total volume of 100 µL. Incubations at 37 °C were terminated after 15 min. For further details see legends to Figures 4 and 5.

Stereospecificity of hydrogen transfer

PPH_4 reductase (1.5 mU) was incubated under standard assay conditions with 2 µM (4R)-[4-^{3}H]NADPH (0.182 µCi) or (4S)-[4-^{3}H]NADPH (0.186 µCi) and a 5-fold excess of substrate. After 10 min incubation at 37 °C the reaction mixture was injected into HPLC and 5 ml fractions were collected. The radioactivity was determined by scintillation counting employing a Packard Tri-Carb spectrometer, model 2450.

HPLC analysis of tetrahydropterins

This was carried out as described by Niederwieser (34). Briefly, tetrahydropterins were separated on a analytical (4.6 × 250 mm) Spherisorb ODS1 (Stagroma AG, 8304 Wallisellen, Switzerland) column connected to an amperometric detector with TL 5A glassy carbon dual flow cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The redox potential was adjusted to 350 mV. As eluent served 6.6 mM Na_2HPO_4, 13.3 mM citric acid, 0.06 mM Na_2 EDTA, 1.4 mM octanesulfonic acid, 0.16 mM dithioerythritol and 3% methanol, at a flow rate of 1 ml/min. The buffer was filtered through a 22 µm filter, flushed with N_2 for 45 min and then degassed by evacuation for 45 min and stored under argon.

Purification of PPH_4 reductase from human liver

The entire purification was carried out at 4–7 °C. Human liver (500 g) was homogenized in 1 L of 10 mM potassium phosphate buffer, pH 7.0, containing $\approx$ 2 mg of phenylmethylsulfonyl fluoride. Homogenization was performed for 3 min and repeated after addition of 1 L of the same buffer using a Virtis-45 homogenizer. After centrifugation (20′500 × *g*, 1 h) the pellet was discarded and the resulting supernatant filtered through cheesecloth. Following ammonium sulfate fractionation (40–60%) the protein precipitate was dialyzed against 10 mM potassium phosphate buffer, pH 6.0, centrifuged (40′000 × *g*, 15 min) and passed through a Sephadex G 25 column (5 × 14 cm, Pharmacia) to remove the residual fat. The protein eluate (222 ml) was applied to a Bio-Gel HTP column (5 × 32 cm, Bio-Rad) equilibrated with 10 mM potassium phosphate buffer, pH 6.0. After washing with 600 ml of the equilibration buffer, a linear gradient (2.4 L, 10 to 300 mM potassium phosphate) was applied (9 ml fractions, flow rate 50 ml/h). The fractions containing PPH_4 reductase were combined and concentrated by Amicon YM 10 ultrafiltration to about 50 ml. The residue was applied to an Ultrogel AcA 54 column (5 × 90 cm, LKB), and eluted with 20 mM potassium phosphate buffer containing 200 mM KCl, pH 7.0 (9 ml fractions, flow rate 70 ml/h). The PPH_4 reductase activity eluted in fractions 103–120. The enzyme solution was concentrated to $\approx$ 10 ml by Amicon YM 10 ultrafiltration, dialysed against 50 mM potassium phosphate buffer, pH 6.2, adjusted to 30% ammonium sulfate, and applied to a column of Fractogel TSK Butyl-650S (2.6 × 28 cm, Merck) equilibrated with 50 mM potassium phosphate buffer, pH 6.2, containing also 30% ammonium sulfate, and washed with 600 ml of the same buffer. The protein was eluted with 1 L of a linear gradient from 30% to

0% ammonium sulfate in 50 mM potassium phosphate buffer while increasing the pH from 6.2 to 7.0 (6 ml fractions, flow rate 120 ml/h). Active fractions were pooled and desalted by repeated dilution and Amicon YM 10 ultarfiltration steps. The concentrated enzyme solution was centrifuged (40'000 × *g*, 15 min) and applied to a Blue Sepharose CL 6B affinity column (1.6 × 6.5 cm, Pharmacia) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The column was washed with 150 ml of this buffer (flow rate 10 ml/h), and elution was carried out with 30 ml of 20 mM potassium phosphate buffer, pH 7.0, containing 100 µM NADPH. The active fractions were combined and concentrated to ≈ 1 ml by Amicon YM 10 ultrafiltration. After buffer exchange (repeated dilution and Centricon 10 (Amicon) filtration steps) the residue (500 µL) was injected onto a Mono Q FPLC column (Pharmacia) equilibrated with 20 mM triethanolamine acetate buffer, pH 7.7. Washing with 3 ml of the same buffer was followed by a linear gradient of 0–500 mM sodium acetate within 14 min (flow rate 1 ml/min). Fractions of 450 µL were collected into tubes which already contained 50 µL of 0.05 M triethanolamine acetate buffer in order to adjust the pH to 7.0. Alternatively, active fractions were rechromatographed on a second, smaller Blue Sepharose column (0.6 × 5 cm). Elution with 150 µM NADPH yielded apparently homogeneous enzyme. The enzyme concentrates, e.g. as obtained from the first Blue Sepharose chromatography, can be stored at −70 °C for several months without significant activity loss. Purest preparations showed rather labile activity.

Miscellaneous methods

The protein concentration was determined following a modification of the method of Bradford (Bio-Rad protein assay kit) using bovine gamma globulin as a standard (35). The purity of the enzyme preparations was checked by SDS/PAGE (36), employing 12.5% acrylamide gels in presence of 0.1% SDS. Proteins were visualized with Coomassie Brilliant Blue R-250. The molecular weight of PPH_4 reductase was estimated by gel filtration on an ultrogel AcA 54 column (5 × 88 cm), equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 200 mM KCl. The column was calibrated with: bovine serum albumin (66 kDa), egg albumin (45 kDa), bovine erythrocyte carbonyl reductase (29 kDa), wale skeletal muscle myoglobin (14 kDa), and horse heart muscle cytochrome c (12.5 kDa). Elution of the marker proteins was monitored by the absorbance at 254 nm. Measurements of the isoelectric point of the enzyme were based on the method of Radola (37), employing Sephadex G 200 super fine flat bed gels containing 10% (by vol.) glycerol and 2% (mass/vol.) ampholyte (pH 4–9) (Serva). Electrophoresis was monitored by the migration of stained standard proteins, using wale skeleton myoglobin (pI 8.3) and horse muscle myoglobin (pI 7.3) from Sigma, cytochrome c (pI 9.4), catalase (pI 5.6) and albumin (pI 4.9) from Serva. After electrofocusing, the gel was sliced into 0.5 cm sections and extracted with 0.2 M Tris/HCl buffer, pH 7.0, for 1 h at 4 °C. The resulting fractions were scanned for PPH_4 reductase activity.

Western blotting

Western blots for glycoprotein detection and antibody probing were performing according to Towbin (38). proteins on nitrocellulose were stained with 0.1% (mass/vol.) amido black 10 B in 25% (by vol.) isopropanol/10% (by vol.) acetic acid for 1 min. In order to determine glycosylation of PPH_4 reductase, the method of Clegg (39) was employed with the exception that Tris buffered saline with bovine serum albumin (10 mM Tris/HCl, pH 7.4, 0.9% (mass/vol.) NaCl, 2% (mass/vol.) bovine serum albumin) as blocking solution and 4-chloro-1-naphthol as color reagent for horseradish peroxidase were used. Washes were without Triton X-100. Transfers intended for antibody probing were developed as described by Gershoni (40). Immunoreactivity of the anitserum (1 : 2'000) was assayed using a commercial goat anti mouse-horseradish peroxidase conjugate (1 : 3'000) and 4-chloro-1-naphthol as color reagent, following the vendor's instructions (Bio-Rad).

Production of a polyclonal antiserum to purified PPH_4 reductase

BALB/c mice were immunized with PPH_4 reductase either purified to apparent homogeneity (described above) or obtained from polyacrylamide gels as described by Tracy (41). For the latter method PPH_4 reductase from the first affinity chromatography step was used. Mixed with complete Frund's adjuvant, 25 µg of the native antigen were injected subcutaneously into 8-week-old animals. 34 days later 20 µg of PPH_4 reductase from Mono Q FPLC in incomplete Freund's adjuvant were inoculated intraperitoneally. In 4-week intervals, two further immunizations were carried out with 8 µg and 15 µg of antigen from SDS/PAGE. After testing the PPH_4 reductase titer, the mouse with the highest production of antibodies was boosted with 25 µg of antigen from SDS/PAGE. One week later the antiserum of this animal was obtained by exsanguination.

Antibody titers were determined by ELISA according to the method of Engvall (42).

Results

Purification of the enzyme, purity and properties

The purification steps are summarized in Table 1. PPH_4 synthase, PPH_4 reductase and sepiapterin reductase from crude liver extract are precipitated at 40 to 60% ammonium sulfate at pH 7.0, while GTP cyclohydrolase is obtained in the 0 to 30% cut. The subsequent Sephadex G 25 chromatography step serves to remove residual fatty material. Hydroxyapatite chromatography is a crucial step since it provides a good separation of PPH_4 synthase, PPH_4 reductase and sepiapterin reductase (Fig. 1). However, complete removal of traces of sepiapterin reductase activity was not achieved before reversed phase chromatography on Butyl Fractogel.

Purification to approximately 80% was achieved by affinity chromatography on Blue Sepharose; repetition of this step yields a single protein band as shown on SDS/PAGE (Fig. 2). All characterization studies were done with enzyme after the first Blue Sepharose chromatography, since PPH_4 reductase is more stable under these conditions, and these fractions were free of other detectable reductase activities. PPH_4 reductase was purified 155-fold with a recovery of 19% taking the hydroxyapatite eluate as 100%. Activity cannot be measured reliably prior to this step, particularly because of the interfering sepiapterin reductase activity (*cf.* above). The addition of bovine serum albumin (0.1 mg/ml) to the purest preparations proved to be useful to stabilize the enzyme activity during storage at −70 °C at least for some weeks, while PPH_4 reductase from the previous step (Blue Sepharose) was stored for 3 months at −70 °C without significant activity loss.

From gel filtration on Ultrogel AcA 54 the molecular weight of PPH_4 reductase was estimated to be 30 kDa, which is about 5 kDa less than the single band

Table 1. Purification of PPH_4 reductase from human liver

Purification stage	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Yield purification	
				%	(-fold)
Crude extract	*	70′560		*	*
Ammonium 40–60% fraction		15′910			
Hydroxyapatite	2′930	990	2.96	100	1
Ultrogel AcA 54	2′420	252	9.6	83	3.2
Tutyl Fractogel	1′600	40	40.0	55	13.5
Blue Sepharose	550	1.2	458.3	19	155

One enzyme unit (U) is defined as the amount of protein producing 1 μmol LPH_4/min from PPH_4 at 37 °C under standard assay conditions. (*) PPH_4 reductase activity was not determined before hydroxyapatite chromatography due to difficulties with sepiapterin reductase catalyzing the same reaction

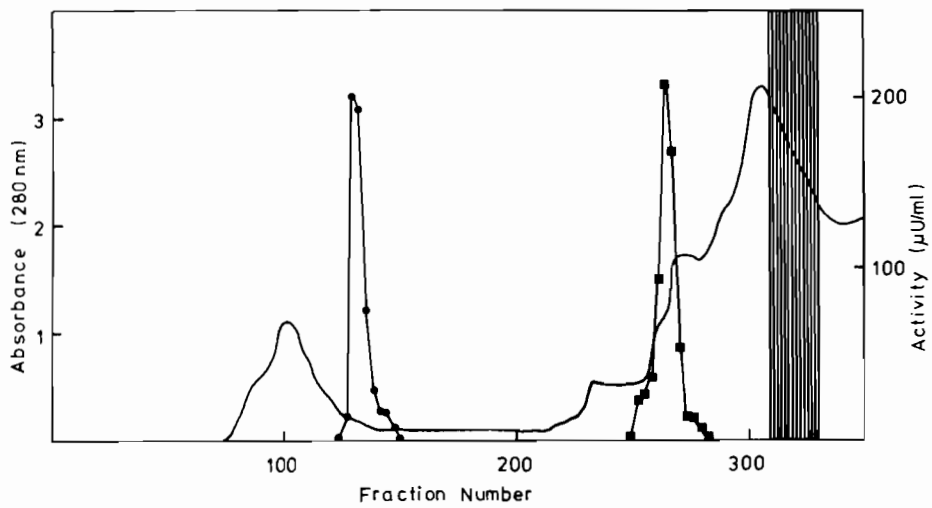


Figure 1. Elution profile of PPH_4 synthase, PPH_4 reductase and sepiapterin reductase from hydroxyapatite column. Protein concentration was measured by absorbance at 280 nm. (–●–●–) PPH_4 synthase activity was determined as described (14). (–■–■–) PPH_4 reductase activity was measured under standard assay conditions. (Striped zone) sepiapterin reductase activity was evaluated qualitatively by the decolorization of sepiapterin (45). The gradient was started at fraction number 106 (9 ml/fraction). End of the gradient was fraction number 375 (not shown)

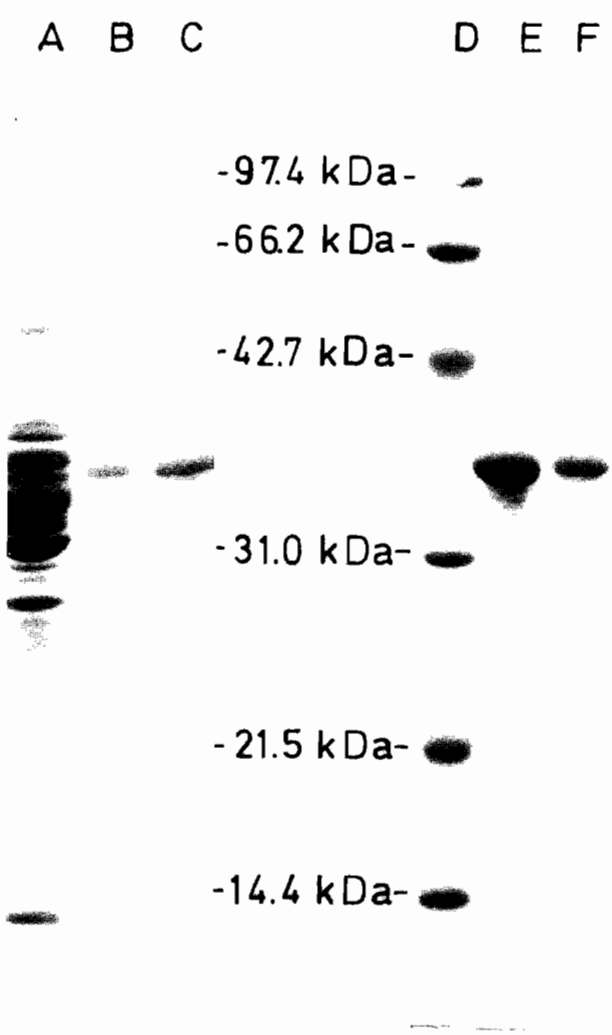


Figure 2. SDS/PAGE (12.5% polyacrylamide) of PPH_4 reductase at different purification steps, and from two distinct preparations: (A) Butyl Fractogel step (≈ 61 µg); (B) blue Sepharose (first chromatography) (≈ 1 µg); (C) Blue Sepharose (rechromatography) (≈ 2 µg); (D) Molecular mass standards; (E) Mono Q FPLC (≈ 14 µg) after chromatography on (F) Blue Sepharose (first chromatography) (≈ 5 µg). Visualization was with Coomassie Brilliant Blue

protein revealed on SDS/PAGE. Aliquots of PPH_4 reductase from Butyl Fractogel chromatography were subjected to isoelectric focusing in a non-denaturating system yielding an average value of pH 5.9 ± 0.1.

Activity measurements

Problems caused by the chemical instability of PPH_4, the natural substrate, and LPH_4, the product, prompted us to examine other compounds as possible alternative substrates for assaying PPH_4 reductase. Though phenylpropanedione, benzyl, phenylglyoxal and diacetyl were slowly reduced by PPH_4 reductase, no suitable substitute for PPH_4 was found. We already reported that sepiapterin and LPH_4 were no substrates of the enzyme (20); 6-(1′-hydroxy-2′-oxo)-propyl-tetrahydropterin also is not a substrate.

An essential parameter for activity measurements is the substrate PPH_4 which must be prepared *in situ* from NH_2TP using PPH_4 synthase. We have set out to optimize this by varying the conditions of formation as shown in Figure 3, in particular by varying the conditions of anaerobiosis or protection by sulfhydril reagents, and the time of incubation. From the results (Fig. 3) it is clear, that radical scavengers are very important as well as the absence of oxygen. The activity of PPH_4 synthase used (10 mU) was chosen as to yield an optimal formation of PPH_4 within the time period required for further manipulations of the sample. The decrease observed at incubation times exceeding 2–3 min probably arises from anaerobic decay of PPH_4 (Fig. 3). It should be pointed out, that at 15 min approximately 2/3 of the product are still present in the mixture under anaerobic conditions, while aerobically formed PPH_4 disappears after 10 min at most.

Since trichloracetic acid interferes with the electrochemical detection it could not be used to terminate PPH_4 reductase reactions. In these cases NaF at a final concentration of 50 mM was used. The activity optimum of the enzyme was at pH 7.0 in potassium

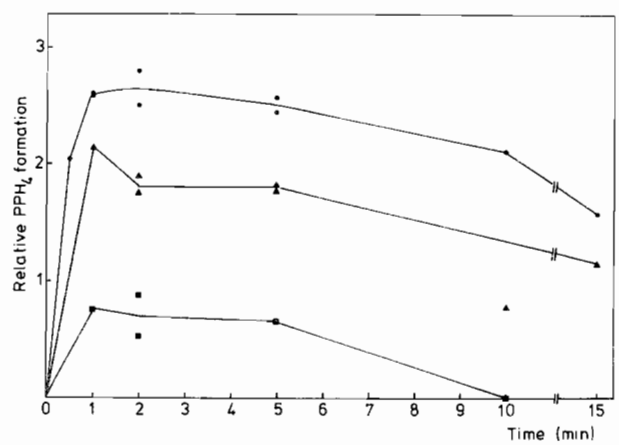


Figure 3. Dependence of PPH_4 formation on anaerobic conditions. PPH_4 formation was assayed in the presence of (-■-■-) 0.25 mM dithioerythritol; (▲ö) 10 mM dithioerythritol and (-●-●-) anaerobically in the presence of 0.25 mM dithioerythritol, 10 mM glucose, 12 U glucose oxidase and 4′800 U catalase. The reaction mixture further contained 0.1 M potassium phosphate buffer, pH 7.0, 8 mM $MgCl_2$, 10 mM PPH_4 synthase and 10 µM NH_2TP. The mixture was first flushed for ≈ 10 s with N_2 in order to remove oxygen from the overhead space of the cuvette, and then kept at 37 °C for the times shown. The reaction was stopped by addition of 0.16 vol. of a 0.2 M EDTA solution. Each sample was analyzed immediately by HPLC with electrochemical detection

phosphate buffer, as shown in Figure 4. Triethanolamine buffer or Tirs/HCl buffer proved to be comparatively less efficient (Fig. 4). From the dependencies shown in Figure 5 some influence of sulhydryl groups on the activity of PPH_4 reductase became obvious. Using different concentrations of dithioerythritol or mercaptoethanol, LPH_4 formation was increased to a maximum at 250 µM dithioerythritol or at 2 mM mercaptoethanol. Higher concentrations particularly of dithioerythritol were detrimental to the activity. This may be attributed to formation of cyclic or intermolecular adducts with the side chain carbonyl functions of PPH_4 or to interaction with the catalytic site of PPH_4 reductase. For these reasons 250 µM dithioerythritol was used throughout for standard assays. Addition of further dithioerythritol to a final concentration of 10 mM upon completion of the reaction proved to be useful in order to stabilize the product. It should be noted that our HPLC assay allows for the determination of PPH_4 in each sample (*cf.* Materials and Methods for details). Thus decay of PPH_4 by e. g. oxidation is in general less than 30% under the conditions employed and over the concentration range used. Taking this substrate loss into account (*cf.* also Materials and Methods), the K_m value for PPH_4 was estimated to be 1.8 µM, and that for NADPH 5.5 µM from the plots shown in Figures 6 and 7.

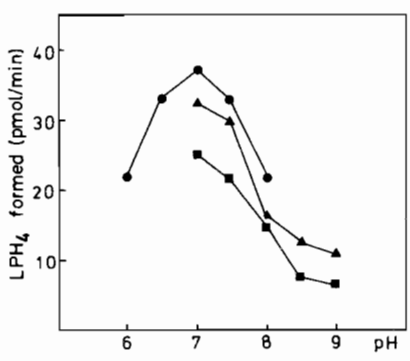


Figure 4. PPH_4 reductase activity as a function of pH and buffer. Activity was determined in potassium phosphate buffer (-●-●-), triethanolamine/HCl buffer (-▲-▲-) and Tris/HCl buffer (-■-■-), at the pH values indicated

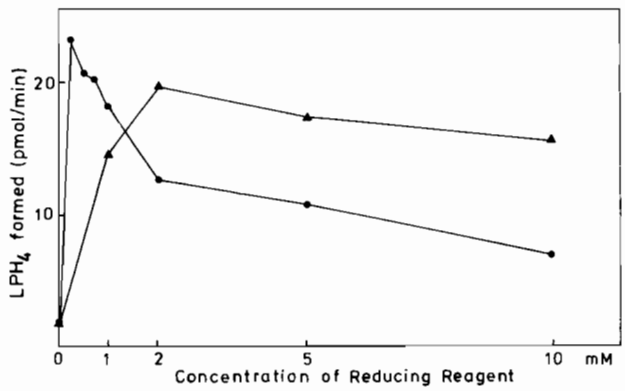


Figure 5. Effect of reducing reagent at various concentrations on LPH_4 formation. PPH_4 reductase activity was measured in presence of dithioerythritol (-●-●-) or mercaptoethanol (-▲-▲-) at the concentrations shown. LPH_4 formation was estimated as described in the text

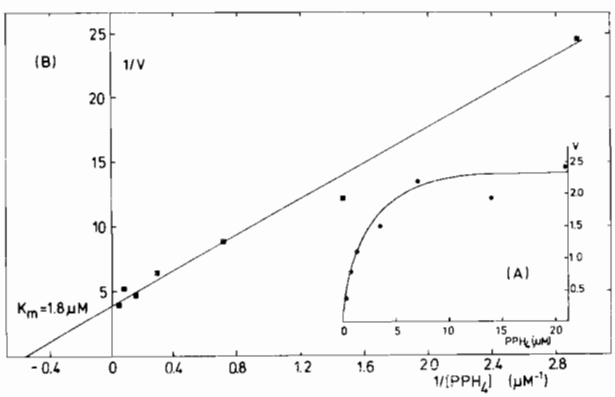


Figure 6. Effect of PPH_4 concentration on the rate of LPH_4 formation. The reaction mixture contained 5 µU PPH_4 reductase, the indicated amount of PPH_4 (after conversion of NH_2TP), 10 mU PPH_4 synthase (salmon liver), 8 mM $MgCl_2$, 0,25 mM dithioerythritol, 2 mM NADPH, 10 mM glucose, 12 U glucose oxidase, 390 U catalase and 0.1 M potassium phosphate buffer, pH 7.0, and was first flushed with N_2 as described in the legend to Fig. 3. The assay volume was 100 µL, the temperature 37 °C and the reaction was terminated after 5 min. (A) linear plot of total LPH_4 formation (µM), (y-scale × 10); (B) double-reciprocal plot of the same data

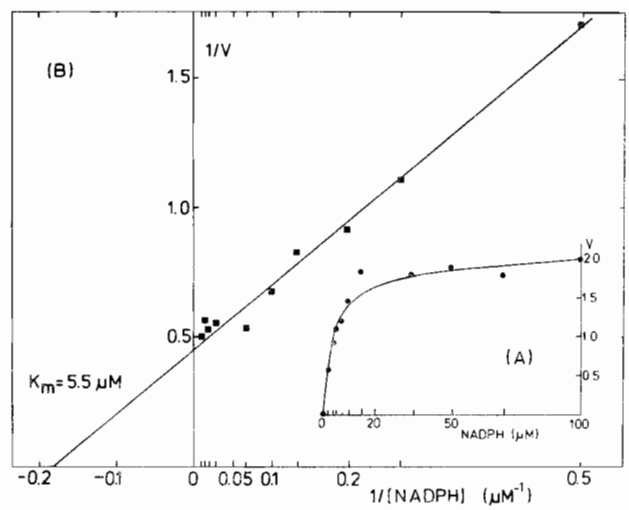


Figure 7. Effect of NADPH on the rate of LPH_4 formation. The reaction mixture contained 20 µU of PPH_4 reductase and the indicated amount of NADPH. Other conditions were the same as described in Fig. 6, except that 20 µM NH_2TP were used. The reaction was stopped after 10 min and LPH_4 formation was analyzed by HPLC with electrochemical detection. (A) linear plot of total LPH_4 formation (µM); (B) double-reciprocal plot of the same data

Stereospecificity of hydrogen transfer

These studies necessarily had to be carried out at relatively low concentrations of labelled NADPH, using excess PPH_4 synthase (5 mU) and PPH_4 reductase (1.5 mU) activities. In two experiments, starting with 153'000 and 135'000 cpm (4R)-[4-^{3}H]NADPH we found 1'550 and 1'490 counts in the LPH_4 fractions obtained from HPLC separations. Using 117'000 and 114'000 cpm (4S)-[4-^{3}H]NADPH in a parallel experiment we found 300 and 290 counts in the corresponding LPH_4 fractions. Thus only $\approx 0.7\%$ of the total tritium label was recovered in the LPH_4 fraction (100% being equimolar transfer excluding isotope effects), and this product was also formed only at 7% of that obtained under standard conditions. These results show an approximately 2.5-fold higher incorporation of label in the LPH_4 peak when (4R)-[4-^{3}H]NADPH was used, compared to the reaction using the (4S)-labeled coenzyme. In spite of the relatively large margin of error, the results suggest that PPH_4 reductase transfers the (4R) hydrogen of NADPH during catalysis. The reasons for the low recovery of radioactivity in the product are not clear at the present. They might be in part due to isotopic selection. A "washing out" of the label due to exchange as in the case e.g. with flavin dependent dehydrogenases seems unlikely, since PPH_4 reductase does not appear to contain such prosthetic groups from its absorption spectrum (not shown).

Polyclonal antiserum to human PPH_4 reductase

Since glycoproteins are known to be highly immunogenic but mostly cause an unspecific immune response to carbohydrates (43), PPH_4 reductase was tested in this context using the concanavalin A assay. This test was negative after Western blot and upon adsorption of the pure enzyme on nitrocellulose. The immunization of BALB/c mice yielded a polyclonal antiserum to PPH_4 reductase with a titer of about 1:15'000 as estimated by ELISA. By use of the immunodot blot (44) a working dilution of 1:2'000 turned out to be suitable for antigen detection on Western blots. The results shown in Figure 8 suggest a specific recognition of the antigen. While amido black stain revealed several protein bands of the enzyme fraction from Butyl Fractogel chromatography, only one of these was located by the antibodies. The position of the band corresponds exactly to the molecular weight of PPH_4 reductase as seen on lane 10, where pure enzyme from Mono Q chromatography was applied. However, the latter also showed minor decay products of the enzyme, which are stained with the antibodies. These protein bands were not detected

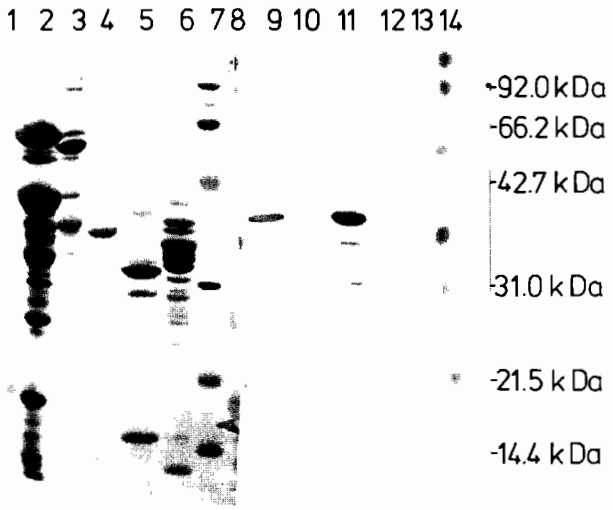


Figure 8. Antibody probing of the polyclonal antiserum produced to purified PPH_4 reductase. Various enzyme preparations were separated on a 12.5% polyacrylamide gel, transferred to nitrocellulose and probed immunologically with diluted antiserum (1:2'000) as described in the text (right hand side of the blot, lane 9 to 14); left hand side: amido black stain of the transferred proteins (lane 1 to 7). From outside to the center: mixture of prestained standard proteins: lysozyme (17 kDa), soybean trypsin inhibitor (27 kDa), carbonic anhydrase (39 kDa), ovalbumin (50 kDa), bovine serum albumin (75 kDa), phosphorylase b (130 kDa) (lanes 1,8 + 14); human liver sepiapterin reductase from early purification steps (lanes 2, 3, 12, 13); PPH_4 reductase after chromatography on Mono Q FPLC (lanes 4 + 11); partially purified prostaglandine-9-keto reductase from pig kidney (lanes 5 + 10); PPH_4 reductase after chromatography on Butyl Fractogel (lanes 6 + 9); mixture of standard proteins containing: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (42.7 kDa), bovine serum albumin (66.2 kDa), phosphorylase b (92 kDa) (lane 7)

in the PPH_4 reductase pool from Butyl Fractogel Chromatography (PPH_4 reductase from Blue Sepharose was not investigated). Cross-reactivity with human liver sepiapterin reductase or pig kidney prostaglandine-9-keto reductase (A. Schieber, unpublished work) was not observed.

Discussion

One objective of our work being the differentiation between sepiapterin reductase and PPH_4 reductase, we first focused our efforts on the quantitative separation of these two reductases in order to facilitate the study of their possible roles *in vivo*. This was achieved by ammonium sulfate fractionation of the crude liver extract, hydroxyapatite chromatography, gel filtration and hydrophobic interaction chromatography on Butyl Fractogel. The final chromatographic steps on Blue Sepharose and Mono Q FPLC yielded

apparently homogeneous PPH_4 reductase which has biochemical properties in close agreement with those published in preliminary form (20, 24). After improving the assay conditions the specific activity of PPH_4 reductase was estimated to be $\approx$ 450 mU/mg protein. This value may be at the lower limit, due to the method, which had to be devised for the generation and stabilization of PPH_4 and LPH_4. An average substrate recovery of $\approx$ 70% also is in line with this assumption.

The activities of PPH_4 reductase and sepiapterin reductase might be differentiated by the stereospecificity of hydrogen transfer. Using purified sepiapterin reductase from rat erythrocytes the latter was shown to be specific for the *pro*-S hydrogen of NADPH (17), while PPH_4 reductase from human liver appears to use the *pro*-R hydrogen of NADPH. This different stereospecificity is of importance since BH_4 formation from NH_2TP catalyzed by partially purified human liver extracts leads to incorporation of the 4-*pro*-S hydrogen of NADPH at each of the C(1′) and C(2′) positions of BH_4 (17). This suggests that sepiapterin reductase is the sole enzyme responsible for PPH_4 reduction. However, these investigations had to be carried out under conditions that were not physiological. The results may therefore not be conclusive with respect to the involvement of PPH_4 reductase in the *in vivo* biosynthetic pathway of BH_4.

So far the strongest support for a physiological role of PPH_4 reductase is given by the high affinity for PPH_4 (K_m = 1.8 μM) and the high rate of substrate turnover (18, 24). Using purified enzymes from rat tissue, Milstien recently presented evidence that, when sepiapterin reductase activity is limiting, BH_4 formation is markedly enhanced by the activity of PPH_4 reductase (23). Unfortunately, no specific inhibitors of either reductase are available yet which might be used for selectively influencing the activity of PPH_4 reductase or sepiapterin reductase in human tissue extracts. The antiserum to human PPH_4 reductase, which specifically recognizes the enzyme in ELISA, on immuno-dot blots and on Western blots, does not cross-react with human sepiapterin reductase. This provides a new experimental tool to localize PPH_4 reductase in human tissues and to investigate the enzyme distribution in correlation with the BH_4 biosynthetic activity of the respective organ. The identity of PPH_4 reductase and aldose reductase, which we have described elsewhere (25) raises some fundamental questions: It should be pointed out, that the *in vivo* biochemical role of aldose reductase is not yet known. This enzyme appears to act on a wide variety of substrates (46), i.e. it has broad specificity. The hypothesis is thus worth considering that the function of this enzyme consists in the oxidoreduction of a variety of carbonyl/hydroxy functional groups, PPH_4 thus being only one of the substrates. This would be a hitherto unconsidered aspect both with respect to the biosynthesis of BH_4 and its regulation, as well as to the metabolism of carbonyls/alcohols.

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