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PURIFICATION OF 6-PYRUVOYL TETRAHYDROPTERIN 2'-KETO REDUCTASE FROM HUMAN LIVER

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Introduction

The biosynthetic pathway of tetrahydrobiopterin (BH_4) (Fig. 1) has been studied intensively by several groups in the last years and most steps have been elucidated (1-6).

The first reaction is the conversion of GTP to dihydroneopterin triphosphate (NH_2TP) catalyzed by a single enzyme, GTP cyclohydrolase I (GTPCH) (7). The nature of the next step, a critical one since it is probably rate-limiting in man, has long remained unclear due to the high instability of the product and the resulting problems of structure elucidation. This key intermediate is 6-pyruvoyl tetrahydropterin (PPH_4) whose structure has by now been well defined using NMR and MS (8,9), as well as other techniques (10,11,12). The elimination of triphosphate and the intramolecular rearrangement are catalyzed by one single enzyme, 6-pyruvoyl tetrahydropterin synthase (PPH_4S), which was purified to homogeneity by Takikawa et al. (13,14). The enzymes which catalyze the two-step reduction of PPH_4 to BH_4 are sepiapterin reductase (SR) and 6-pyruvoyl tetrahydropterin reduc-

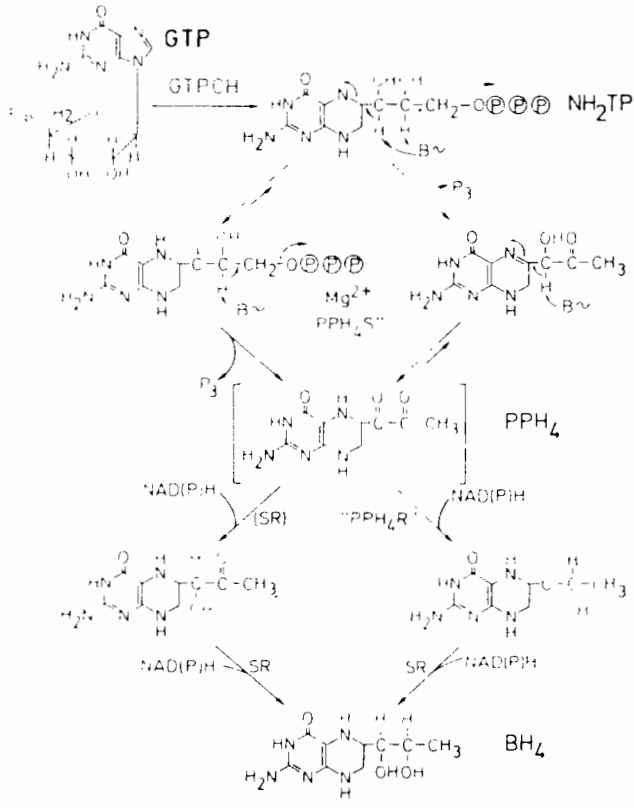


Fig. 1. Proposed pathway for the biosynthesis of tetrahydrobiopterin from guanosine triphosphate in human liver.

tase (PPH_4R), respectively. The occurrence of the second reductase, PPH_4R, was described by several groups, e.g., Milstien et al. in rat and bovine adrenal medulla (15), Switchenko et al. in Drosophila heads (10), Masada et al. in silk worm (5), and Takikawa et al. from our group in human liver (14). This enzyme was named 6-lactoyl-PtH_4 synthase (15), sepiapterin synthase B (10), and sepiapterin synthetizing enzyme 2 (SSE_2) (5).

It is not yet clear whether PPH_4R is on tne BH_4 biosynthetic pathway *in vivo*. Neither has the sequence of the two-step reduction, i.e., whether

reduction occurs first at C-1' or at C-2', been elucidated yet. For the study of this question N-acetyl serotonin, a potent inhibitor of SR, has been used (16). However, we have observed that in contrast to SR from rat tissues which is completely inhibited (16), this reagent inhibits human liver SR to about 70% only, and, in addition, PPH_4R is partially inhibited.

In collaboration with M. Masada from Tokyo Metropolitan University we have studied the SR-mediated reduction of synthetic aromatic substrates carrying a 1',2'-dicarbonyl side chain. It appears that the reduction of the 1'-carbonyl group precedes that of the second carbonyl group. As was shown by Masada et al. (5) and also by our group, SR has to be present in large excess in order to guarantee efficient BH_4 biosynthesis (1,17).

B. Wermuth (18) has reported the isolation of an NADPH-dependent carbonyl reductase from human brain and liver, which reduces a number of biologically and pharmacologically active carbonyl compounds. Using Wermuth's pure enzyme, we have confirmed that this carbonyl reductase is different from PPH_4R and is not involved in the biosynthesis of BH_4 (Masada et al., in preparation).

In this communication we outline the purification procedure of PPH_4R from human liver and describe some of its properties. The availability of pure enzyme is important in order to clarify the role of PPH_4R in the biosynthetic pathway in vivo.

Materials and Methods

Human liver was obtained from traffic accident victims. BH_4 and sepiapterin were purchased from Dr. Schircks Laboratories (Buechstrasse 17a, 8645 Jona, Switzerland). NH_2TP, 6-(1'-oxo-2'-hydroxypropyl)-5,6,7,8-tetrahydropterin and 6-(1'-hydroxy-2'-oxopropyl)-5,6,7,8-tetrahydropterin were prepared as described by Takikawa (13). N-acetyl serotonin and dihydrofolate reductase were from Sigma, NADPH and $NADP^+$ from Boehringer Mannheim. Other chemicals were obtained from commercial sources. PPH_4S and SR were

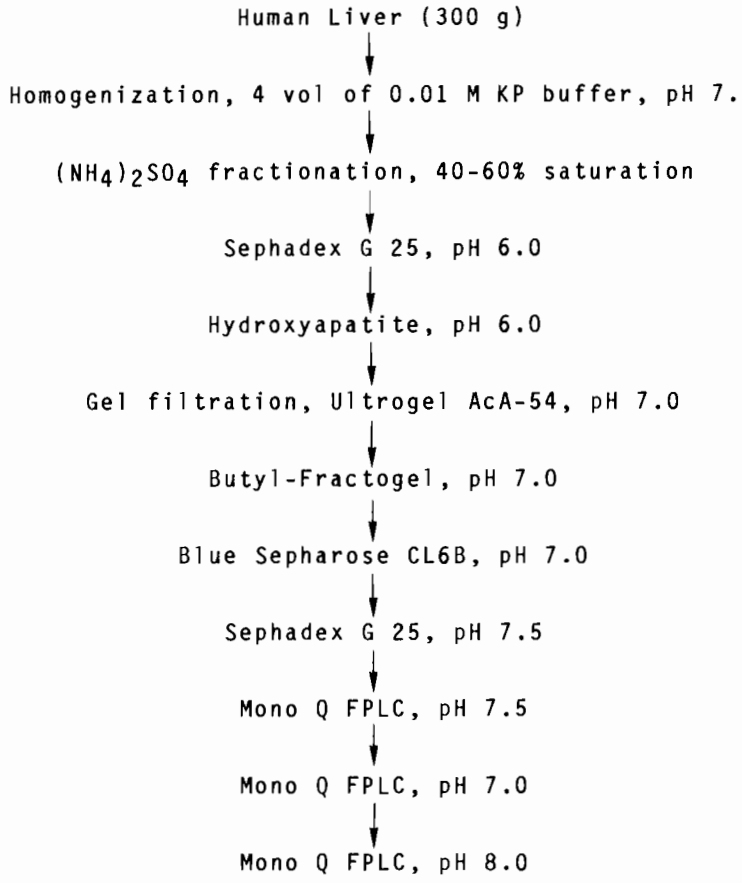
purified as described by Hasler (19) and Takikawa (13). The detailed purification procedure of PPH_4R from human liver will be described elsewhere (Curtius et al., in preparation).

Protein was determined with the Bio-Rad protein assay kit using bovine gamma globulin as standard. PAGE was according to Laemmli (20) (12.5% acrylamide, 0.1% SDS, visualization by silver staining). The molecular mass of the enzyme was also estimated by gel filtration (Ultrogel ACA 54, equilibrated with 20 mM KP buffer, pH 7.0, containing 200 mM KCl; markers: bovine serum albumin (66 kD), egg albumin (45 kD), bovine erythrocyte carbonyl reductase (29 kD), wale skeletal muscle myoglobin (14 kD), and horse heart muscle cytochrome c (12.5 kD), elution monitored by A_{280nm}). Glycoprotein detection was according to Clegg (21), however, using 4-chloro-1-naphtol as horseradish peroxidase color development reagent. The isoelectric point was determined as described by Radola (22).

Assays for PPH_4R and SR. The assay mixture (100 µl) for PPH_4R activity contained 20 µM NH_2TP, 100 mM Tris/HCl, pH 7.4, 8 mM $MgCl_2$, 1mM DTE, 1 mM NADPH, 2 mU PPH_4S from salmon liver obtained after gel filtration (19), and the enzyme fraction. The formed product, 6-(1'-oxo-2'hydroxypropyl)-5,6,7,8-tetrahydropterin, was measured by electrochemical detection using HPLC (24). In the presence of SR traces identified by 6-(1'-hydroxy-2'-oxopropyl)-5,6,7,8-tetrahydropterin and/or BH_4 production (electrochemical detection), PPH_4R was assayed with 1 mM N-acetyl serotonin. SR activity was essentially determined as described by Katoh (23).

Results and Discussion

The procedure for the purification of PPH_4R is outlined in Scheme 1. Table 1 gives the activity and recovery data of a typical purification. Because of the presence of SR PPH_4R activity can be measured only after the hydroxyapatite step when SR is largely removed. The assay for PPH_4R activity is not possible in the presence of SR since the latter also reduces PPH_4. On the other hand, N-acetyl serotonin inhibits human SR only par-



Scheme 1. Procedure for purification of 6-pyruvoyl tetrahydropterin reductase from human liver.

tially. In order to approximate the actual PPH_4R activity before SR is completely removed, N-acetyl serotonin is used in assay mixtures with only slight SR contamination.

The hydroxyapatite column chromatography was found to be essential since it provides separation from SR and also good recovery of PPH_4S. After the AcA 54 gel chromatography SR was essentially removed. Gel filtration on Ultrogel AcA 54 allows estimation of the molecular weight of PPH_4R which is about 30'000 D.

Table 1: Purification of PPH_4R from Human Liver

Procedure	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Recovery (%)	Purification (-fold)
Crude extract	1'065	*	44'400		*	*
Ammonium sulfate 40-60% fraction	100		9'400			
Hydroxyapatite	53	135.0	600	0.23	100	1
Ultrogel AcA 54	16	122.4	150	0.82	91	3.6
Butyl Fractogel	2.6	62.0	17.7	3.50	46	15.2
Blue Sepharose	1.1	38.0	0.3	126.60	28	550.4

*PPH_4R activity cannot be determined reliably before the hydroxyapatite column step due to the presence of SR which catalyzes the same reaction.

After Blue Sepharose affinity chromatography the increase of specific activity was about 550-fold. This purification factor does not take into account the first two purification steps which lead to a 100-fold purification, approximately. The total amount of protein was 0.3 mg from 300 g of human liver corresponding to an overall recovery of 28%. After the Blue Sepharose step some contaminating proteins were still present (see SDS-PAGE in Fig. 1). These were eliminated by threefold FPLC chromatography on Mono Q ionic exchanger. This resulted in two fractions (detection at 280 nm), both of them showing PPH_4R activity. While the first peak was still contaminated with minor protein impurities, the second yielded a single band corresponding to a molecular weight of approx. 35'000 D on

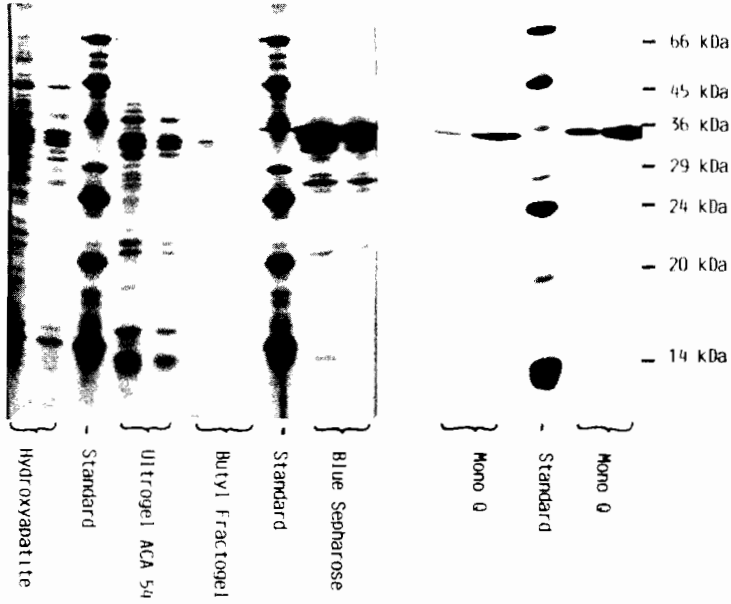


Fig. 2. SDS-Page with 12.5% polyacrylamide. Left gel: enzyme preparation from different purification steps, as mentioned in the figure; right gel: active fractions from the third Mono Q run. The mixture of standard proteins contained: bovine albumine (66 kD), egg albumine (435 kD), glycerinaldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), α-lactalbumin (14 kD).

SDS-PAGE (Fig. 2). Since this corresponds to the molecular weight determined by using gel filtration, it must be concluded that the enzyme is a monomer.

Analytical isoelectric focusing (pH range 4-9, Servalyt T 4-9) on G-200 superfine revealed an isoelectric point of pH 6.0 ±0.2. Glycoprotein detection using Concanavalin A and peroxidase was negative. In the presence of 10 mM DTE less activity was found than with a 1 mM solution, suggesting that PPH_4R is not particularly sensitive to -SH group oxidation. Only PPH_4 and not 6-(1'-oxo-2'-hydroxypropyl)-5,6,7,8-tetrahydropterin or sepiapterin is a substrate for this enzyme. Most probably 6-(1'-hydroxy-2'-oxopropyl)-5,6,7,8-tetrahydropterin will also turn out to be a substrate for this enzyme, however, so far this has not been investigated in detail. This indicates a much higher specificity of PPH_4R compared to SR, the latter catalyzing the reduction of a variety of diketo functions (25). It is, therefore, reasonable to assume that this new enzyme indeed plays a role in BH_4 biosynthesis, although this still remains to be proven by direct experiments.

A particular problem is posed by nomenclature. The name "6-pyruvoyl tetrahydropterin reductase" might not be appropriate, since SR also reduces 6-pyruvoyl tetrahydropterin, although with a different preference for the side chain carbonyl groups. "6-pyruvoyl tetrahydropterin 2'-keto reductase" or "6-pyruvoyl tetrahydropterin 2'-carbonyl reductase" might be better choices.

The use of specific inhibitors for PPH_4R and of antibodies which are currently in preparation might help to elucidate the question whether this enzyme is on the biosynthetic pathway or not, and to solve the sequence problem mentioned above.

Conclusion

An enzyme which catalyzes the reduction of the 2'-keto group of 6-pyruvoyl tetrahydropterin, the intermediate in tetrahydrobiopterin biosynthesis, has been purified from human liver to apparent homogeneity. The enzyme has a native molecular weight of approx. 35'000 D and is a monomer. It apparently contains no carbohydrates and has an isoelectric point of pH 6.0. In the presence of NADPH this enzyme efficiently catalyzes the formation of 6-(1'-oxo-2'-hydroxypropyl)-5,6,7,8-tetrahydropterin from 6-pyruvoyl tetrahydropterin. 6-(1'-oxo-2'-hydroxypropyl)-5,6,7,8-tetrahydropterin and sepiapterin are no substrates.

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