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Introduction

The biosynthetic pathway of tetrahydrobiopterin (BH_4) has been studied intensively by several laboratories in the recent past. Dihydroneopterin triphosphate (NH_2TP) has been recognized to be a key intermediate, its formation from GTP being catalyzed by a single enzyme, GTP cyclohydrolase I (see [1] for review). The nature of the next step, a critical one since it is probably rate limiting in man, has long remained elusive due to the high instability of the product and the resulting problems in elucidating its structure. A breaktrough has been achieved recently and almost simultaneously in several laboratories working along independent lines [2-6]. It consists in the realization that the postulated intermediate should have a tetrahydropterin structure (see [7] for discussion). This would differentiate it in terms of the redox state of the pterin from the dihydro structure of the starting material NH_2TP; however, it would have the same oxidation state of the pterin in the product BH_4. In earlier work it was shown that a purified enzyme from human liver [8] as well as a similar preparation from Drosphila melanogaster [2] is effective in catalyzing the elimination of triphosphate from NH_2TP. These intriguing properties in addition to the obvious biochemical and physiological relevance prompted us to attempt the purification from human liver of the first enzyme involved in the conversion of NH_2TP to BH_4. An enzyme having similar catalytic properties has been purified recently by Switchenko and Brown [2] from Drosophila heads. In this report we provide a short review of the recent work on the biosynthesis of BH_4, focusing on the human system, and we describe some of the properties of the enzyme which we succeeded in purifying to apparent homogeneity from human liver. For a detailed description of the catalytic function and of the physicochemical properties

Table 1: Purification of PPH_4S

Preparation steps	Volume ml	Total protein mg	Total activity[a] mU	Specific activity mU/mg	Recovery %	Purification factor
Crude extract	1,890	72,500	50.0	0.0007	100	1.0
Ammonium sulfate, 35-55 % fraction	280	11,600	45.5	0.004	91	5.7
Hydroxyapatite eluate	400	230	43.5	0.19	87	269
Heat-treated hydroxyapatite eluate	40	49	42.0	0.86	84	1,224
Ultrogel AcA 44 eluate	160	1.7	36.0	21.2	72	30,252
DEAE-Fractogel 650 S eluate	70	0.3	30.0	100.0	60	143,000

The purification was started with 500 g aliquots of human liver.

[a] One unit of activity is defined as the amount of enzyme which produces 1 μmole of BH_4/min from NH_2TP at 37°C under standard assay conditions.

Adapted from [10]. We refer to the original literature for details.

of the enzyme we refer to the detailed publications [9,10].

Properties of 6-Pyruvoyl-Tetrahydropterin Synthase (PPH_4S)

This enzyme has now been purified approx. 140'000 fold from human liver with an unusually high recovery of about 60% [10]. The sequence of the purification steps is summarized by the data in Table 1. Two steps are of crucial importance in this procedure: The one is chromatography on hydroxyapatite (Fig. 1), which was carried out at an early stage since it allows a complete separation from sepiapterin reductase (SR) and from the second pteridine dependent reductase (cf. below), besides providing excellent purification. This enables the purification of these three enzymes from the same batch of human liver. The second important step is heat treatment, which takes advantage of the remarkable heat stability of PPH_4S and allows the elimination of proteins which were found to interfere with the activity tests. It should be noted that the enzyme at the purification level shown in the last line in Table 1 still consists of two bands, as revealed by SDS-PAGE [9,10]. Using conventional chromatographic techniques, we did not succeed in purifying the enzyme to homogeneity. However, upon gel electrophoresis under nondenaturing conditions, the major band was resolved as an active protein which was then shown to be apparently homogeneous by SDS-PAGE [10]. PPH_4S is a tetrameric enzyme with a subunit M_r of approx. 19 kD and it probably contains carbohydrates. It is very stable when stored in frozen state; however, it loses up to 80% of its activity in the absence of sulfhydryl reagents. This loss of activity is reversible upon treatment with DTE. Recently, this enzyme has been called 6-pyruvoyl-tetrahydropterin synthase [11], a name which better describes its catalytic activity compared to other proposals.

Catalytic Functions of 6-Pyruvoyl-Tetrahydropterin Synthase

The homogeneous enzyme requires Mg^{2+} for catalytic activity, shows saturation kinetics using NH_2TP as a substrate, and has a K_m value of 10 μM. The catalytic activity was monitored either by following BH_4 production by HPLC in a combined assay containing excess SR and NADPH or by monitoring 6-pyruvoyl-tetrahydropterin (PPH_4) directly [9,10]. The enzyme catalyzes the conversion of the dihydropterin chromophore of NH_2TP to a chromophore which is characteristic of pterins in their tetrahydro state [12]. This is analogous

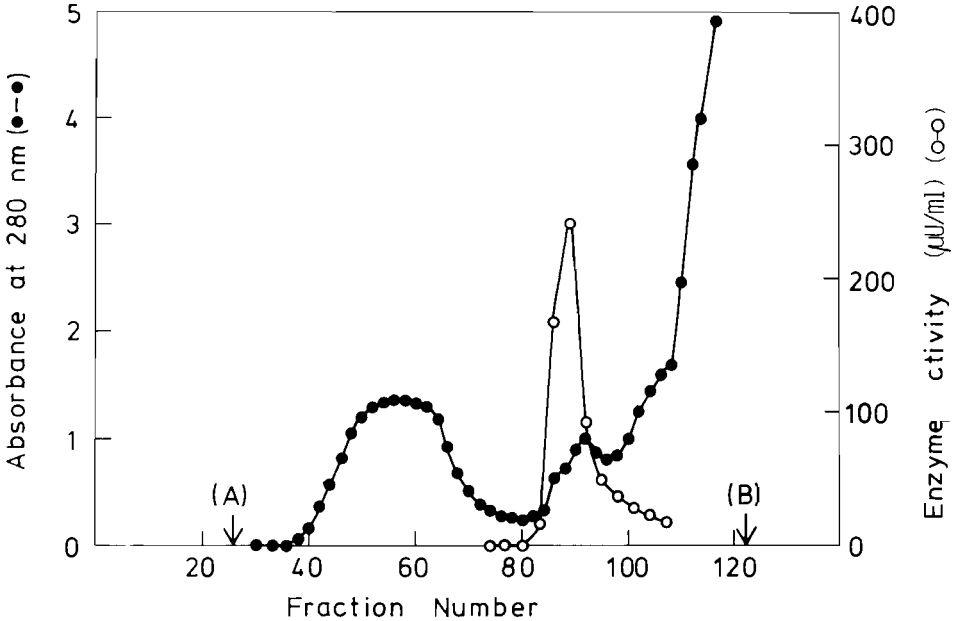
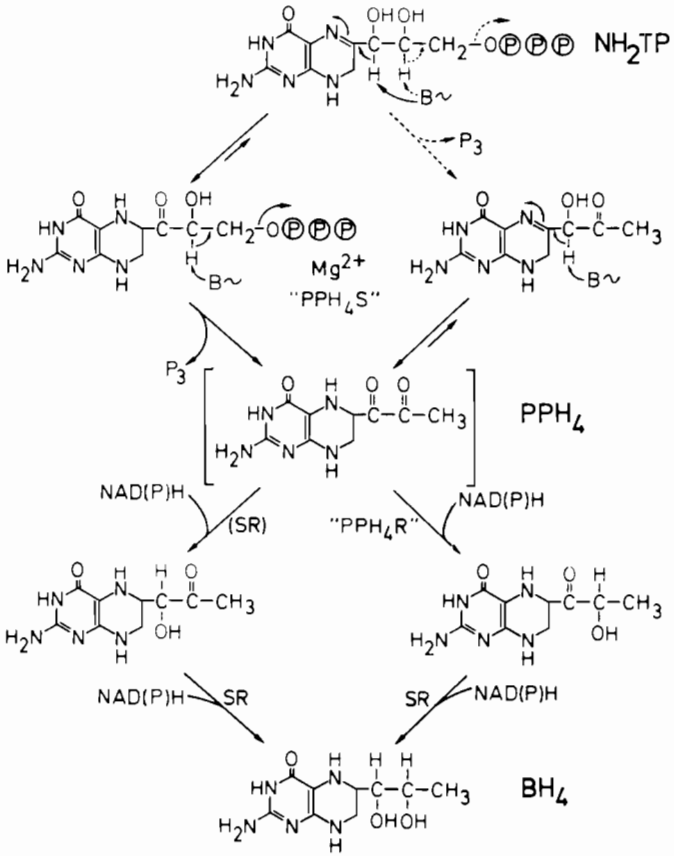


Fig. 1. Purification of PPH_4S by chromatography on hydroxyapatite column. —●—: protein concentration measured by UV absorbance at 280 nm. —○—: enzyme activity. Arrow (A) indicates the starting point of gradient of phosphate buffer, and arrow (B) indicates the end of gradient. SR and PPH_4 reductase elute at fraction 120. (adapted from [10]).

to the findings of Switchenko and Brown [2] which were interpreted similarly. The enzyme also catalyzes the elimination of triphosphate from NH_2TP [8]. These two steps lead via ketonization of the intermediate enolic form to a tetrahydropterin species having a diketo side chain at position 6. The chemistry of these events is shown in Scheme 1. A still open question, the solution of which appears experimentally quite difficult, is the sequence of the two events leading to PPH_4. The two alternatives are shown on the left hand and on the right hand side, respectively, of Scheme 1. From a chemical point of view, both pathways have their own merits. If elimination of triphosphate were to occur first (Scheme 1, right hand side), the C(1')-H function in the intermediate shown would be acidified considerably. This would promote the next step, the rearrangement to form the tetrahydropterin structure, since



Scheme 1. Biosynthetic pathway of BH_4 from NH_2TP.

the latter involves abstraction of the C(1') hydrogen. The same arguments hold also for the left hand side alternative since in the tetrahydro intermediate, having the C(1') keto function, C(2')-H is similarly acidified, facilitating triphosphate elimination. It is also likely that in both cases the second step will be faster than the first one due to the effects mentioned. Conceivably, the same active center base might function in abstracting both

hydrogens as protons, and the intermediate is not released from the active center during conversion of NH_2TP to BH_4. The ^1H-NMR experiments detailed elsewhere in this volume do not indicate substantial differences in the rates of appearance of signals of the product and the disappearance of the C(1')-H and C(2')-H signals of NH_2TP [13].

Evidence for the Structure of 6-Pyruvoyl-Tetrahydropterin

Elucidation of the structure of the product formed from NH_2TP in the presence of PPH_4S has been exceedingly difficult due to its high instability. In fact, PPH_4 has also been named "dispropterin" (greek: "the hidden one") [4]. On the one hand, during its formation triphosphate is eliminated and tetrahydropterin chromophore is formed without the requirement of exogenous redox equivalents; the structure shown in Scheme 1 thus would be the most logical one. On the other hand, the following evidence has been accumulating recently which supports this structure proposal: When the conversion of NH_2TP catalyzed by PPH_4S is carried out in D_2O, one equivalent of deuterium is incorporated at position C(6) [3,14]. Reduction or trapping of the intermediate with (^2H)-NADH or $NaBD_4$ leads to incorporation of 2 equivalents of deuterium in the side chain [3,14,5], thus demonstrating the presence of two keto functions. The conditions used routinely for the detection of PPH_4 in HPLC analysis are typical for tetrahydropterins [15]. When the conversion of NH_2TP is followed by ^1H-NMR in D_2O, the resonances attributed to the hydrogens at C(1') and C(2') disappear, while that of the C(7)-H_2 is shifted upfield [13]. Furthermore, a new signal appears at a field strength compatible with the presence of a -CO-CH_3 group. An explanation for this apparent discrepancy is given by the mass spectral analysis of the products obtained in D_2O buffer which shows that approx. 75% of the hydrogens at C(3') have been exchanged with solvent, most probably by the action of PPH_4S [13]. Finally, FAB mass spectra of PPH_4 isolated by chromatography on C-18 cartridge shows the correct molecular ion [16]. The observation that PPH_4 elutes as a relatively broad peak in most HPLC systems [10], remains puzzling. This could suggest the occurrence of isomeric forms at equilibrium which might arise from interaction of the diketo side chain with the tetrahydropterin nucleus (ring closure?).

Requirement of Further Enzymes for the Formation of BH_4 from PPH_4

The conversion of PPH_4 to BH_4 requires the introduction of 2 hydride equivalents at positions 1' and 2' which are provided by NADPH. SR is able to catalyze the reduction of both functions [4,17]. However, large excesses of this enzyme are required for efficient conversion, indicating that a further enzyme might play a role. In fact, in the side fractions obtained during the purification of PPH_4S, a reductase activity (PPH_4R) was found in addition to SR, which efficiently catalyzes the reaction of NADPH with PPH_4. This enzyme has an approx. M_r of 35 kD and catalyzes the reduction of the C(2')=O function to yield SH_2 (dihydrosepiapterin or 6-lactoyl-tetrahydropterin; cf. Scheme 1, lower right hand side). In contrast to SR [17], this enzyme is not inhibited by N-acetylserotonin. Dihydrosepiapterin and sepiapterin are not substrates for this enzyme. This indicates a much higher specificity compared to SR which catalyzes the reduction of a variety of diketo functions [17]. From this evidence it is reasonable to assume that this new enzyme in-

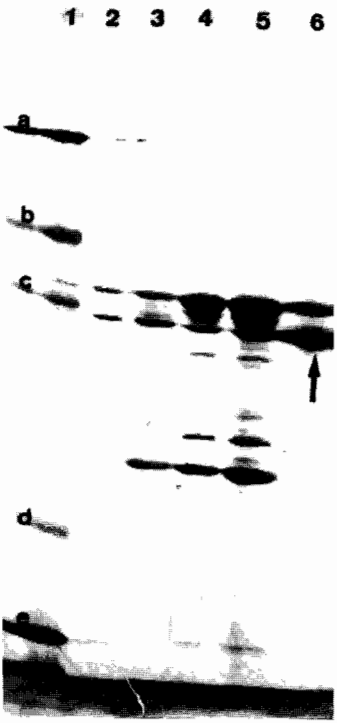


Fig. 2. SDS-Page with 12% polyacrylamide. Slot 1: mixture of standard proteins; a albumin, bovine (MW = 66.000 D), b albumin, egg (MW = 45.000 D), c glyceraldehyde-3-phosphate dehydrogenase (MW = 36.000 D), d trypsin inhibitor (MW = 20.100 D), e α-lactalbumin (MW = 14.100 D). Slots 2-6: PPH_4-reductase (PPH_4R) from human liver after homogenization, ammonium sulfate fractionation (40-60%), chromatography on hydroxyapatite, Ultrogel AcA-44, DEAE-Fractogel 650 S, and Blue Sepharose. Slots 2+3: PPH_4R from Blue Sepharose eluted with low, and slots 4-5 with high NADPH concentration. Slot 6: PPH_4R after re-chromatography on Blue Sepharose.

deed plays a role in BH_4 biosynthesis. We have purified the enzyme to approx. 60% and the last fraction showed 2 major bands as shown by the SDS-PAGE depicted in Fig. 2.

Conclusion

The experimental evidence presently available can be interpreted by the sequences shown in Scheme 1. Thus, efficient conversion of NH_2TP to BH_4 requires three enzymes in the human liver system, i.e., PPH_4S, PPH_4 reductase, and SR, while a single protein, PPH_4S, catalyzes the formation of the intermediate PPH_4. This might be significantly different in other organisms such as, e.g., D. melanogaster or silk worm. The structure of the intermediate PPH_4 now appears to be established in its essence. On the other hand, details of its properties, in particular of its chemical reactivity (stability), still await elucidation. The question as to which of the enzymes mentioned is rate limiting in the overall process also still has to be answered and might require detailed kinetic analysis. This point is of relevance in view of the different cases of genetical diseases described in recent years which involve BH_4 biosynthesis.

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