REVIEW ARTICLE

Tetrahydrobiopterin biosynthesis, regeneration and functions

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Tetrahydrobiopterin (BH_4) cofactor is essential for various processes, and is present in probably every cell or tissue of higher organisms. BH_4 is required for various enzyme activities, and for less defined functions at the cellular level. The pathway for the *de novo* biosynthesis of BH_4 from GTP involves GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase. Cofactor regeneration requires pterin-4a-carbinolamine dehydratase and dihydropteridine reductase. Based on gene cloning, recombinant expression, mutagenesis studies, structural analysis of crystals and NMR studies, reaction mechanisms for the biosynthetic and recycling enzymes were proposed. With regard to the regulation of cofactor biosynthesis, the major controlling point is GTP cyclohydrolase I, the expression of which may be under the control of cytokine induction. In the liver at least, activity is inhibited by BH_4, but stimulated by phenylalanine through the GTP cyclohydrolase I feedback regulatory protein. The enzymes that depend on BH_4 are the phenylalanine, tyrosine and tryptophan hydroxylases, the latter two being the rate-limiting enzymes for catecholamine and 5-hydroxytryptamine (serotonin) biosynthesis, all NO synthase isoforms and the glyceryl-ether mono-oxygenase. On a cellular level, BH_4 has been found to be a growth or proliferation factor for *Crithidia fasciculata*, haemopoietic cells and various mammalian cell lines. In the nervous system, BH_4 is a self-protecting factor for NO, or a general neuroprotecting factor via the NO synthase pathway, and has neurotransmitter-releasing function. With regard to human disease, BH_4 deficiency due to autosomal recessive mutations in all enzymes (except sepiapterin reductase) have been described as a cause of hyperphenylalaninaemia. Furthermore, several neurological diseases, including Dopa-responsive dystonia, but also Alzheimer's disease, Parkinson's disease, autism and depression, have been suggested to be a consequence of restricted cofactor availability.

Key words: cyclohydrolase, dehydratase, dihydropteridine, nitric oxide, 6-pyruvoyl-tetrahydropterin, sepiapterin.

INTRODUCTION

Since the review on the biosynthesis and function of tetrahydrobiopterin (BH_4) by D. S. Duch and G. K. Smith in 1991 [1], tremendous efforts have been made in an attempt to understand the molecular basis of its biosynthesis and regeneration, and of some important regulatory processes. Today, all corresponding human genes for the BH_4 metabolic pathway have been cloned, and the structures of the enzymes have been solved for at least one organism. Furthermore, knowledge of the crystal structures and detailed kinetic analyses have enabled us to make precise proposals for the complex organic chemistry reactions. Although there are still many open questions, the role of BH_4 in living organisms is undisputed. In particular, in the areas of medicine concerned with inherited metabolic disorders and cardiovascular and other nitric oxide-dependent processes, interest in the role of BH_4 continues to grow, and we are on the way towards the first attempts at gene therapy.

In this review, the main focus will be on the structure and function of the mammalian enzymes responsible for the synthesis and regeneration of BH_4.

BIOSYNTHESIS OF BH_4

In the following, the *de novo* biosynthesis and recycling of the BH_4 cofactor is described. The 'salvage' pathway that feeds BH_4 from dihydrobiopterin via NADPH-dependent dihydrofolate reductase does not seem to have any compensatory role. At least in the case of BH_4 deficiency observed in newborn animals (see below), this 'alternative' pathway is not sufficient to compensate for the defects in biosynthesis (or recycling).

Reaction mechanism of the *de novo* pathway

BH_4 biosynthesis proceeds via the *de novo* pathway in a Mg^{2+}-, Zn^{2+}- and NADPH-dependent reaction from GTP via two intermediates, 7,8-dihydroneopterin triphosphate (H_2NTP; compound **5** in Scheme 1) and 6-pyruvoyl-5,6,7,8-tetrahydropterin (PTP; compound **8**). These intermediates have been isolated, although they are rather unstable. The three enzymes GTP cyclohydrolase I (EC 3.5.4.16; GTPCH), 6-pyruvoyl-tetrahydropterin synthase (EC 4.6.1.10; PTPS) and sepiapterin reductase (EC 1.1.1.153; SR) are required and sufficient to carry out the proper stereospecific reaction to 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (Scheme 1, compound **11**). With the crystallographic structures, including the characteristics of the active centres of all three enzymes, having been elucidated, the essential information for the interpretation of the reaction mechanism is available. Moreover, NMR studies on the reaction mechanisms of all three enzymes have revealed the details of the hydrogen transfer process and the stereochemical course of the reactions [2].

Abbreviations used: qBH_2, quinonoid dihydrobiopterin; BH_4, tetrahydrobiopterin; DCoH, dimerization cofactor of hepatocyte nuclear factor 1α; DHPR, dihydropteridine reductase; GFRP, GTP cyclohydrolase I feedback regulatory protein; GTPCH, GTP cyclohydrolase I; HNF-1α, hepatocyte nuclear factor 1α; H_2NTP, 7,8-dihydroneopterin triphosphate; NOS, nitric oxide synthase (the prefixes i, n and e denote the inducible, neuronal and endothelial isoforms respectively); PAH, phenylalanine 4-hydroxylase; PCD, pterin-4a-carbinolamine dehydratase; PTP, 6-pyruvoyl-5,6,7,8-tetrahydropterin; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase.

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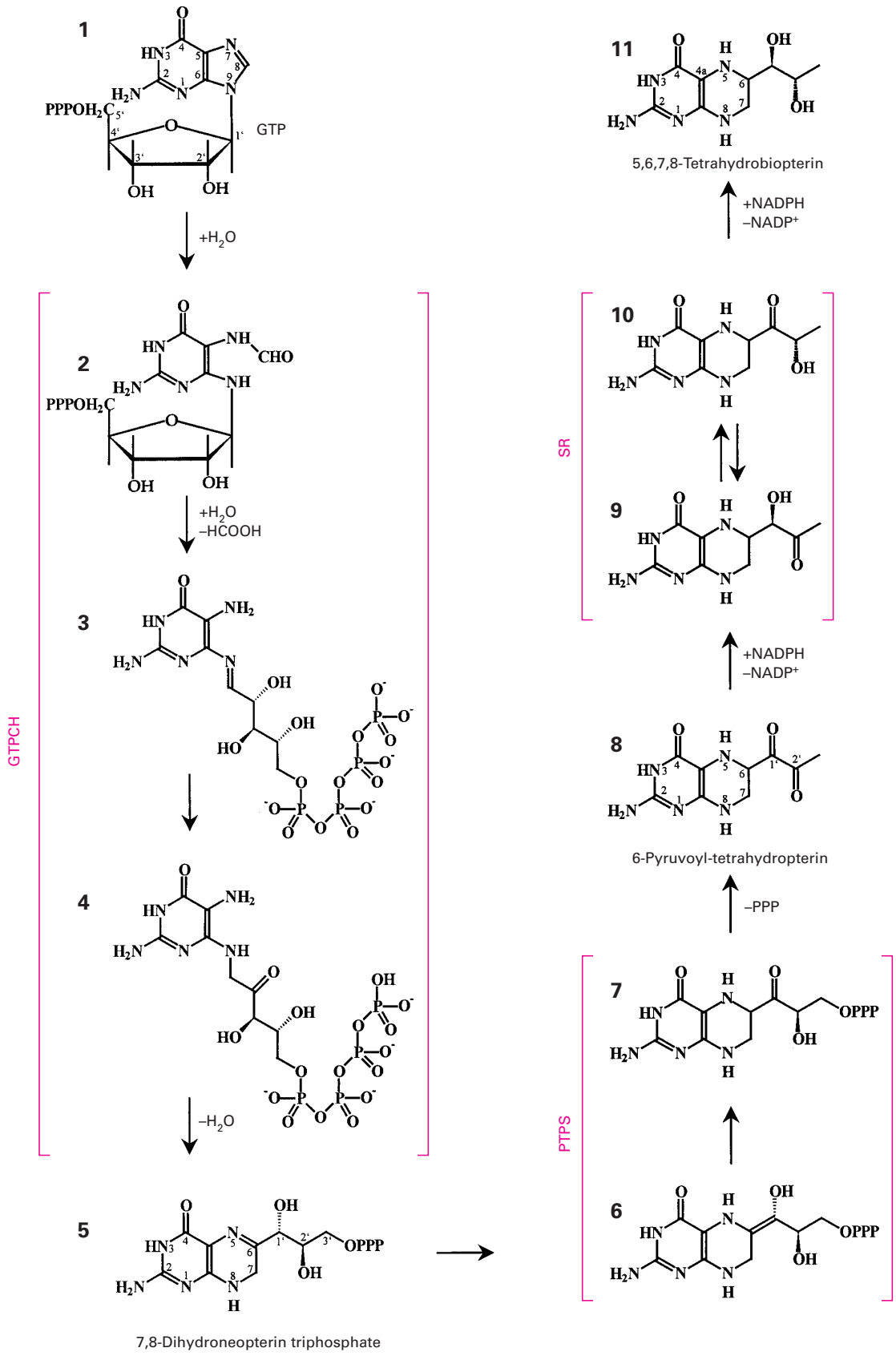


Scheme 1 Reaction mechanism for the *de novo* pathway for BH_4 biosynthesis

See the text for details.

The committing step of the reaction is carried out by GTPCH, a homodecamer consisting of a tightly associated dimer of two pentamers [3]. GTPCH contains 10 equivalent active centres with pockets 10 Å (1 nm) deep. The interface of three subunits, two from one pentamer and one from the other, forms an active site. This cavity is structurally stabilized by two loops (residues 109–113 and 150–153 in the *Escherichia coli* enzyme) formed by hydrogen-bond interactions and a salt bridge (Glu-111–Arg-153). An intramolecular disulphide bridge (Cys-110–Cys-181) is not essential for active-site integrity, as shown by Cys-mutant structures (G. Auerbach, unpublished work). The atomic structure of this pocket is not only highly selective for GTP, but also provides residues for complete charge compensation in order to render obsolete Mg^{2+}-assisted binding to the protein, as found in other nucleoside triphosphate binding proteins.

A catalytic mechanism was proposed based on structural analysis obtained from *E. coli* GTPCH co-crystals with the dGTP analogue and several active-site mutants [4] (see Scheme 1). The purine hydrolysis reaction may be initiated by protonation of N-7 by a specific histidine residue (His-179 in the *E. coli* enzyme). Attack by water at C-8 and subsequent opening of the imidazole ring results in a first intermediate, the 2-amino-5-formylamino-6-ribofuranoside triphosphate (Scheme 1, compound **2**). Protonation of the bridging O atom in the furanose ring and release of C-8 of GTP by another histidine residue (His-112 in *E. coli*) as formate yields the Schiff-base intermediate (compound **3**). The subsequent Amadori rearrangement catalysed by involving the γ-phosphate of GTP and a serine residue (Ser-135 in *E. coli*), and keto-enol tautomerization, results in the intermediate compound **4**. The last step is the ring expansion by closing between N-7 and C-2′, yielding the product H_2NTP (compound **5**). It is assumed that this final reaction step takes place at the protein surface or in solution after dissociation, as the spatial structure of the active-site pocket does not favour the reaction occurring inside.

The reaction from H_2NTP (compound **5**) to PTP (compound **8**) is catalysed by PTPS in a Zn^{2+}- and Mg^{2+}-dependent reaction without the consumption of an external reducing agent (Scheme 1). This conversion involves a stereospecific reduction by an internal redox transfer between atoms N-5, C-6 and C-1′, oxidation of both side-chain hydroxy groups, and an unusual triphosphate elimination at the C-2′–C-3′ bond in the side chain. Crystallographic analysis revealed that PTPS is composed of a pair of trimers arranged in a head-to-head fashion to form the functional hexamer [5]. The homohexamer contains six active sites that are located on the interface of three monomers, two subunits from one trimer and one subunit from the other trimer. The catalytic centre and the reaction mechanism have been studied by crystallographic and kinetic analysis of wild-type and mutant PTPS enzymes from the rat [6]. In addition, the crystal structure of the inactive mutant Cys-42 → Ala PTPS in complex with its natural substrate H_2NTP has been determined [7]. Each catalytic centre harbours a Zn^{2+}-metal-binding site in a 12 Å deep cavity. The active-site pocket with the specific pterin-anchoring Glu residue for salt-bridging plus two hydrogen-bonding amino acids appears to be similar to the equivalent sites in GTPCH, SR, dihydroneopterin epimerase and neopterin aldolase. The active-site pocket contains, in addition, two catalytic motifs: a Zn^{2+}-binding site and an intersubunit catalytic triad formed by a Cys, an Asp and a His residue. The tetravalent co-ordination of the transition metal is accomplished via the Nϵ-atoms of three His residues and a fourth ligand provided by the side chain moiety of the H_2NTP substrate. Unfortunately, neither the triphosphate nor the putative Mg^{2+} ion moieties could be defined in the electron-density map. Zn^{2+} plays a crucial role in catalysis, as it activates the protons of the substrate and stabilizes the intermediates (Scheme 1, compounds **6** and **7**). The proposed reaction mechanism is the following. Protonation of N-5 and abstraction of a proton from the C-1′ side-chain carbonyl atom leads to N-5–C-6 double-bond reduction (compound **6**). Stereospecific protonation of C-6 and oxidation of C-1′–OH to C-1′=O gives compound **7**. The last step is the abstraction of a proton from the C-2′ carbon of the carbonyl side chain, followed by triphosphate elimination and tautomerization to yield PTP (compound **8**). The Cys residue (Cys-42 in the rat enzyme) of the catalytic triad appears to be the general base for stereospecific protein abstraction in both reaction steps.

The final step is the NADPH-dependent reduction of the two side-chain keto groups of PTP (Scheme 1, compound **8**) by SR. The overall structure of SR is a homodimer stabilized by a common four-helix bundle [8]. Each monomer contributes two α-helices to the central dimerization domain and forms a separate complex composed of seven parallel β-sheets surrounded by α-helices. The C-terminal end of the β-sheets contains, in close proximity, NADPH and the pterin-binding site, the latter comprising a 15 Å deep pocket. The pterin substrate is anchored by the guanidino moiety of a specific Asp residue (Asp-258 in mouse SR; Asp-258 is also the anchoring residue for *N*-acetyl-5-hydroxytryptamine inhibitor binding; see below). The C-1′ carbon of the pterin side chain is in direct proximity to NADPH and a Tyr hydroxy group (Tyr-171 in the mouse SR). This Tyr is the central active-site residue for optimal proton transfer [9]. Based on kinetic, crystallographic and NMR data, the initial step is the NADPH-dependent reduction at the side-chain C-1′-keto function, leading to the formation of 1′-hydroxy-2′-oxopropyl tetrahydropterin (compound **9**) [2]. Internal rearrangement of the keto group via side-chain isomerization leads to the 1′-keto compound 6-lactoyl tetrahydropterin (compound **10**). Intermediate compound **10** is then reduced to BH_4 (compound **11**) in a second NADPH-dependent reduction step. While the pterin substrate remains bound to the active site, the redox cofactor has to be renewed after the first reduction. It is thus assumed that $NADP^+$ is exchanged at the opening located at the opposite side of the pterin-binding and entry pocket.

Besides its involvement in the *de novo* biosynthesis of BH_4, SR may also participate in the pterin salvage pathway by catalysing the conversion of sepiapterin into dihydrobiopterin, which is then transformed into BH_4 by dihydrofolate reductase [10].

Enzyme structures

GTPCH

Structural crystallographic data are available for GTPCH from *E. coli* [3,4]. The homodecameric GTPCH is composed of two dimers of pentamers (Figure 1). Each subunit contains 221 amino acids and folds into an $\alpha+\beta$ structure with a predominantly helical N-terminus. The N-terminal antiparallel helix pair α_2/α_3 is remote from the rest of the molecule. The compact C-terminal body of the monomer (residues 95–217) is formed by a central four-stranded antiparallel β-sheet (β-1 to β-4) that is flanked on both sides by α-helices (α_4, α_5 and α_6) (Figure 2). The N-terminal helix α_1 lies on top of helices α_4 and α_5. The association of two GTPCH monomers to form dimers is driven by the formation of a four-helix bundle by helices α_2 and α_3. Five monomers interact along their β-sheets to form a 20-stranded antiparallel β-barrel with a diameter of 35 Å. The decamer is formed by a face-to-face association of two pentamers, whereby the antiparallel helix pair α_2/α_3 of one monomer is intertwined with those of another monomer. The decamer has a toroidal

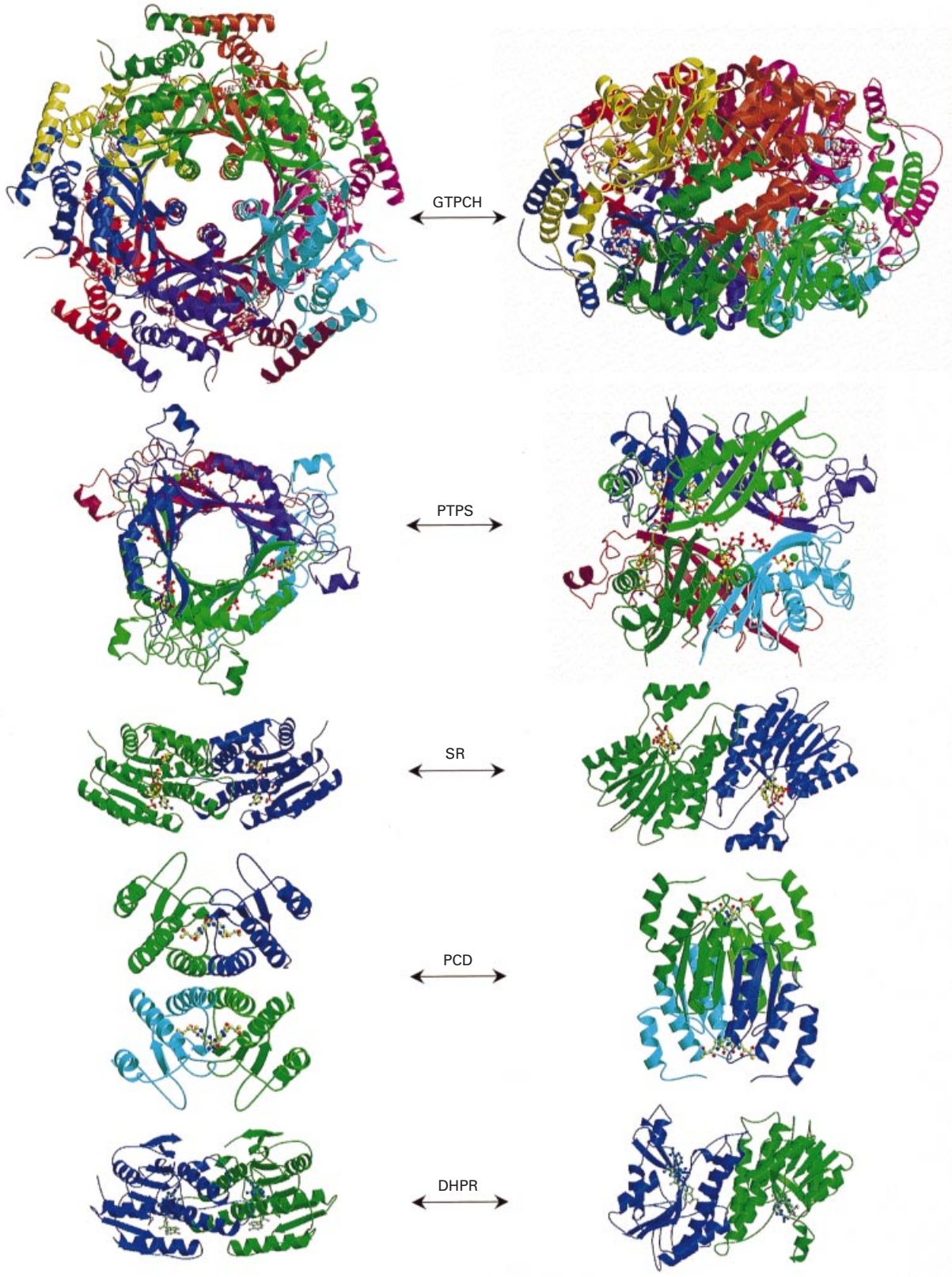


Figure 1 Three-dimensional structures of BH_4-metabolizing enzymes

Shown are ribbon-type representations of the main-chain foldings of the enzymes involved in *de novo* BH_4 biosynthesis and BH_4 regeneration. Substrates are shown in ball-and-stick representation, with atoms in standard colours. On the right side the enzymes are shown rotated by 90° around the *x*-axis. The crystal structure co-ordinates used are: GTPCH from *E. coli* (Protein Data Bank entry code 1GTP) [3], PTPS from rat liver (1GTQ, 1B66) [7,137], SR from mouse (1SEP) [8], PCD/DCoH from rat (1DCH, 1DCP) [58,138], and DHPR from rat liver (1DHR) [65]. The figure was prepared using the programs MOLSCRIPT [139] and RENDER [140].

shape with an approximate height of 65 Å and a diameter of 100 Å. It encloses a cavity of 30 Å × 30 Å × 15 Å that is accessible through the pores formed by the five helix bundles in the centre of the pentamers, but has no opening at the decamer equator. The active site is located at the interface of three subunits, two from one pentamer and one from the other. There are thus 10 equivalent active sites per functional unit.

Sequence information for a number of GTPCHs from diverse organisms is available (Figure 2a). High overall sequence similarity is found among the mammalian enzymes (approx. 90% identity). A comparison of all sequences reveals that mainly the C-terminal sequence is evolutionarily conserved, i.e. the C-terminal 120 residues of the human and *E. coli* enzymes are 60% identical. Moreover, almost all residues participating in pterin binding and/or catalysis appear to be conserved. This high sequence identity suggests that the tertiary and quaternary structures of GTPCH enzymes are most probably very similar. The high diversity of the N-termini between the mammalian and the non-vertebrate enzymes, most notably the N-terminal extension in the *Drosophila* sequence, may be due to different regulatory functions, such as, for instance, docking sites for the GTP cyclohydrolase I feedback regulatory protein (GFRP) (see also below). The N-terminal part of the multimeric enzyme may be exposed to the protein surface in all species, as has been shown for the *E. coli* enzyme, thus allowing putative protein–protein interaction(s).

PTPS

Only the recombinant rat liver enzyme has been crystallized to yield interpretable diffraction data (residues 7–144) [6]. Each subunit folds into a compact, single-domain $\alpha+\beta$ structure. The monomer consists of a sequential, four-stranded, antiparallel β-sheet (β-1 to β-4) with a 25-residue helix-containing (α_A) insertion between β-1 and β-2 at the bottom of the molecule (Figure 2). Strands 3 and 4 are connected via an α-helical turn. A segment between strands 2 and 3 forms a pair of antiparallel helices, α_B and α_C, layered on one side of the four β-sheets. Three monomers assemble into a trimer, forming a 12-stranded antiparallel β-barrel structure surrounded by a ring of α-helices. Crystallographic and experimental data have revealed that the mammalian PTPS is homohexameric and dissociates into trimers (Figure 1). Two trimers arrange in a head-to-head fashion to form a barrel, the functional PTPS hexamer with an overall shape of 60 Å × 60 Å × 60 Å. Due to the relatively tilted order of the β-sheet, the pore in the trimer is conically shaped with a diameter of 6–12 Å. In the hexameric enzyme, the pore has a smaller opening towards the trimer interface, and opens up also equatorially to the hexamer surroundings. The inside of the barrel accumulates a cluster of basic and aromatic residues stretching radially into the pore. Each subunit of the homohexamer contains one putative active site that is located at the interface of three monomers, two subunits from one trimer and one subunit from the other trimer. Each active site harbours three histidine residues (His-23, His-48 and His-50 in the rat enzyme), the Nϵ-atoms of which co-ordinate the binding of the Zn^{2+} metal. In the unliganded state, a water molecule is bound as the fourth ligand. In a complex structure with substrate, C1′ and C2′ side-chain hydrolysis of the dihydroneopterin ligand displaces the bound water, yielding a pentavalent co-ordination of the transition metal [7].

Complete sequences for PTPS enzymes from several mammals and from *Drosophila* are available, while partial amino acid sequences from salmon are also known (Figure 2b). With the exception of the C-terminal extension in the fly enzyme, the overall amino acid identity is around 80%. All residues involved in substrate binding and catalysis are conserved among these sequences. The high degree of subunit conservation between the species implies that all of these PTPS enzymes form a homohexameric structure.

SR

The 1.25 Å crystal structure of mouse SR in complex with $NADP^+$ has been solved [8]. The 261 amino acids of the monomer fold into a single-domain α/β-structure. A seven-stranded parallel β-sheet, β_A–β_G, in the centre of the molecule is sandwiched by two arrays of three α-helices (α_C, α_B, α_G and α_D, α_E, α_F) (Figure 2). The association of two monomers to the active homodimeric SR (Figure 1) leads to the formation of a four-helix bundle (helices α_E and α_F of each monomer). Due to the two-folded crystallographic symmetry of the homodimeric molecule, the parallel β-sheet in monomer A is in an antiparallel orientation relative to the β-sheet of monomer B, enclosing an angle of 90°. The overall dimensions of the SR dimer are 40 Å × 50 Å × 80 Å. The two substrate pockets bind sepiapterin (or 6-pyruvoyl-tetrahydropterin; compound **8** in Scheme 1) and the cofactor $NADP^+$/NADPH from opposite sides to the enzyme.

Amino acid sequence comparison for the available SRs has revealed a high degree of similarity among the mammalian enzymes (around 90%), as well as a high degree of conservation compared with the fish and fly enzymes (Figure 2c). It is thus anticipated that the tertiary and quaternary structures of SR are conserved throughout these species.

From both amino acid sequence and three-dimensional structure, SR can be assigned to the family of short-chain dehydrogenases/reductases. These enzymes all use NAD(H) or NADP(H) as the cofactor, and contain a strictly conserved Tyr-Xaa-Xaa-Xaa-Lys sequence motif (Tyr-171 to Lys-175 in the mouse SR sequence).

Comparison of all three biosynthetic enzymes

There is no sequence identity among the three BH_4 biosynthetic enzymes. However, a comparison of polypeptide secondary structure has revealed that the C-terminal domain of GTPCH shows a similar subunit fold to that of PTPS, and also to neopterin aldolase, H_2NTP epimerase and uroate oxidase, whereas the overall structure of SR is entirely different [11,12]. The tertiary structures of GTPCH and PTPS revealed in both enzymes a central pore for which the function is still unknown; it seems not to be directly involved in catalysis and substrate binding. A speculative but attractive role for these cavities might be 'channelling' of the unstable pterin intermediates from one enzyme to the other (pterin compounds **5** and **8** in Scheme 1). A potential substrate channelling would imply that the biosynthetic enzymes must interact and thus form some type of 'super complex'. Such physical interaction has not yet been observed. In terms of complex-formation with their natural substrates, all three enzymes show the same active-site architecture. Furthermore, this pterin-binding motif unit is quite similar to the GTP-binding motif in small GTP-binding proteins [8].

Genes encoding the biosynthetic enzymes

The corresponding genes for all three enzymes are known from human and mouse, in some instances also from other organisms. An overview of gene names, chromosomal location and exon content for the human genes is presented in Table 1.

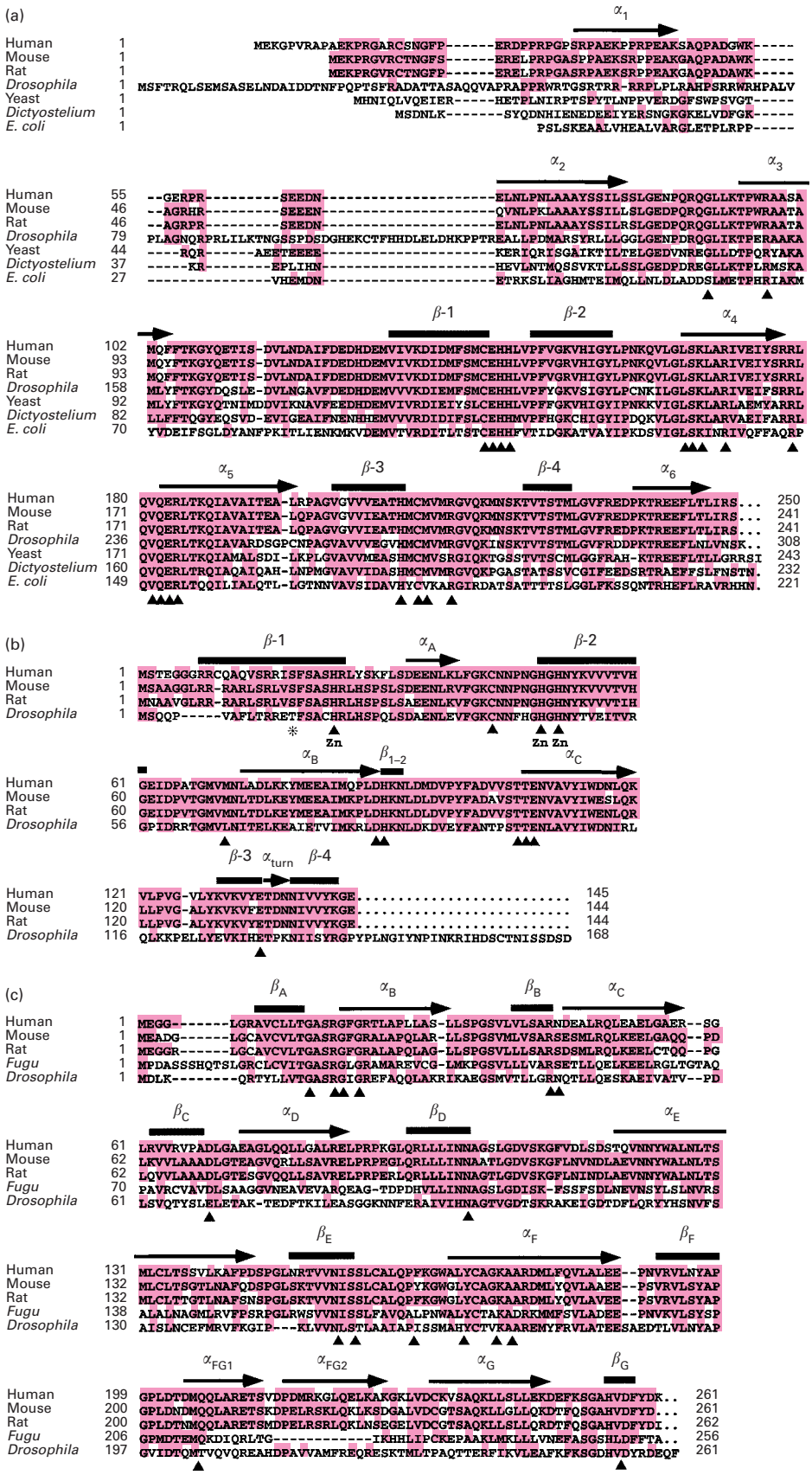


Figure 2 For legend see facing page.

Table 1 Human BH_4-metabolizing enzymes

For references, see the text. Gene names have been approved by the HGMW nomenclature committee.

Enzyme (EC number)	Gene	Chromosomal location	No. of exons	No. of amino acid residues	Size (kDa × no. of subunits)
GTPCH (3.5.4.16)	*GCH1*	14q21.1–22.2	6	250	27.9×10
PTPS (4.6.1.10)	*PTS*	11q22.3–23.3	6	145	16.4×6
SR (1.1.1.153)	*SPR*	2p13	3	261	28.0×2
PCD (4.2.1.96)	*PCBD*	10q22	4	103*	11.9×4†
DHPR (1.6.99.7)	*QDPR*	4p15.3	7	244	25.8×2

* In liver, the protein was found to lack the starting methionine residue.

† Homotetrameric (α_4) only as a carbinolamine dehydratase; heterotetrameric ($\alpha_2\beta_2$) in complex with HNF-1α.

GCH1

Human and mouse GTPCHs are encoded by a single-copy gene, *GCH1*, which is composed of six exons spanning approx. 30 kb [17]. The five introns in the mouse and human genes are at identical sites. In the fruit fly *Drosophila*, GTPCH is encoded by the *Punch* locus, and three out of four introns have identical positions (introns 2–4 [12a]), whereas the single intron present in the *Dictyostelium discoideum* gene is inserted at a different site [12b]. The yeast gene has no intronic sequences [12c]. Alternative splicing has been observed for human exons 5 and 6, and *Drosophila* exon 1. Three types of GTPCH cDNAs with different 3′ ends have been isolated from human liver [13]. Type 1 cDNA, with 250 codons, has the longest coding region and the greatest similarity to those reported from rat and mouse [14,15]. Furthermore, the full-length type 1 GTPCH cDNA has been isolated from a pheochromocytoma cDNA library [16]. Alternative usage of the splice acceptor site within exon 6 generates the shorter type 2 mRNA [17]. Type 3 mRNA contains, besides exons 1–5, an extension of exon 5 due to non-usage of the splice sites from 'intron 5'. Putative proteins derived from type 2 and 3 mRNAs lack the C-terminal 40 amino acids containing residues involved in pterin binding and catalysis, and are highly conserved compared with the *E. coli* enzyme (see Figure 2a). In agreement with this is the observation that individual expression of recombinant proteins from these cDNAs in bacterial cells yielded GTPCH activity only with the type 1 cDNA. *Drosophila* contains two 5′ splice variants for the GTPCH mRNA. These alternative exons confer distinct N-terminal domains to each predicted protein, which cannot be aligned with the N-termini of any other GTPCH. It was speculated that different GTPCH isoforms in *Drosophila* and in higher eukaryotes might have specific subcellular and/or tissue distributions, and that alternative isoforms could associate with and respond to different regulatory or modifying proteins. So far there is no experimental evidence for such regulatory diversity. However, it was observed that two different complexed forms of GTPCH activity exist in human liver, which can be separated by molecular mass (400 kDa and 600 kDa). In this case both forms contained the same protein subunit with identical mass, as judged by SDS/PAGE analysis [18].

The transcription start site of the mouse *GCH1* gene and a limited analysis of the promoter sequences of the human and mouse genes are available [17,19]. Corresponding CCAAT boxes were found to be conserved between the mouse and human promoters. However, the sequences did not reveal typical sites known to be involved in interferon-γ signal transduction, nor was any response observed upon interferon-γ treatment of transfected human cells with corresponding reporter constructs. Since it is well established that GTPCH gene expression can be induced by interferon-γ in various rodent and human cells (see below), further studies are necessary in order to characterize the mammalian *GCH1* promoter.

PTS

The organization of the human and mouse *PTS* genes is highly conserved [19a,37,38]. They both span a region of 6–7 kb of genomic DNA and contain six exons, with their introns at identical positions. Besides a splicing polymorphism that occurs in at least some human cell types, leading to skipping of exon 3 and aberrant protein expression, all cells and tissues investigated so far appear to express the same functional human PTPS mRNA. The human, but not the mouse, genome contains in addition a retropseudogene, *PTS-P1*, located on chromosome 9q13. In this retropseudogene, the 5′ 25 codons and an internal fragment of 23 bp region corresponding to exon 3 (codons 54–61) are entirely absent. The overall similarity to the 3′ portion of the PTPS cDNA is 74%. The *Drosophila purple* gene produces two PTPS mRNAs: a head-specific one containing three introns and expressed from a proximal promoter, and a constitutive mRNA with an additional intron in the 5′ non-translated region expressed from a distal promoter [19b]. The reading frame encoding PTPS from both mRNAs is the same. When compared with the human and mouse *PTS* intron–exon organization, the first two introns in the *Drosophila* gene coding sequence have identical positions, and the third *Drosophila* intron is located two codons upstream of the corresponding human gene intron 5 position.

No information is thus far available regarding the transcriptional start sites and corresponding promoter analyses for the mammalian *PTS* genes.

SPR

The genomic organization of the mouse and human *SPR* genes encoding SR is very similar [20,21]. They both span a region of 4–5 kb and the reading frames are split into three exons. No alternative splice variants have been observed. Only the mouse harbours a genomic pseudogene (*Sprp*), which contains exons 1 and 2 plus the intervening and partial flanking sequences for these two exons, with an overall similarity to the functional *SPR* gene of 82%.

Transcriptional start sites (+1) have been determined for the *SPR* genes from both species [20,21]. No TATA-like sequences and no CAAT-box motifs were found in the upstream vicinities

Figure 2 Amino acid sequence alignments of the BH_4-synthesizing enzymes (a) GTPCH, (b) PTPS and (c) SR

Alignments were generated using the Clustal W program [141]. Identical residues and residues with functional similarities (V/L/I, R/K, M/L, D/E) are highlighted. Residues involved in catalysis and/or substrate binding are marked with a triangle. The identified phosphorylation site in PTPS (Ser-19 in the human sequence) is marked with an asterisk. The accession numbers for the amino acid sequences for (**a**) GTPCH are NM000161 (human), L09737 (mouse), J05729 (rat), AF159422 (*Drosophila*), CAA87397 (yeast), Q94465 (*Dictyostelium*), X63910 (*E. coli*); for (**b**) PTPS are Q03393 (human), AAD15827 (mouse), P27213 (rat), P48611 (*Drosophila*); and (**c**) SR are P35270 (human), Q64105 (mouse), P18297 (rat), AAC60297 (*Fugu*) and AAD12760 (*Drosophila*).

of the +1 sites. Furthermore, for the mouse *SPR*, fusion studies with a reporter gene were conducted, revealing that the promoter contains a sequence between −83 and −51 bp upstream of the transcriptional start site that is essential for expression.

CpG abundance

An elevated abundance of the dinucleotide CpG in a DNA sequence of at least 200 bp spanning exon 1 and the transcriptional start site of a gene in comparison with the residual DNA sequence is known to be typical of housekeeping genes (C+G content > 50%, frequency of observed versus expected CpG = 0.6; [21a]). Such a CpG content analysis with the human and mouse BH_4 biosynthetic genes revealed, as expected, that *GCH1* is a regulated gene, whereas *PTS* and *SPR* are predicted to be constitutively expressed (conducted with the CpGPlot program of Gardine-Garden and Frommer [22]; M. Turri and B. Thöny, unpublished work). However, more detailed analyses are required in order to better define the promoters of the mammalian BH_4 biosynthetic genes.

Regulation of enzyme expression and activity

Regulation of BH_4 biosynthesis appears to be complex, and an integrated picture of the signal transduction and control pathways does not yet exist. Depending on the cell or tissue type, all enzymes are thought to be constitutively expressed, such as in the liver or in some brain regions. In other tissues, enzyme expression can be induced or is completely absent. Expression of GTPCH at least is inducible, and PTPS activity can be elevated to some extent. Moreover, post-translational modification(s) of all three biosynthetic enzymes and regulation of GTPCH by the GFRP may modulate enzymic activities.

GTPCH

The committing step for BH_4 biosynthesis is the major controlling point for cofactor biosynthesis. GTPCH activity can be regulated at the transcriptional and post-translational levels and by the GTPCH-interacting protein GFRP, which modulates enzyme activity. Regulation of the level of transcription by cytokines, phytohaemagglutinin and endotoxin (lipopolysaccharide) in a cell- and tissue-specific manner is probably predominant. Cytokines such as interferon-γ, tumour necrosis factor-α, stem cell factor (or kit ligand) and interleukin-1β, or a specific combination of these, induce GTPCH gene expression *in vitro* and/or *in vivo* in various cells, including T-lymphocytes, macrophages, monocytes, fibroblasts, bone-marrow-derived mast cells and mesangial cells. Some of these stimulatory agents, e.g. lectin, may act in an indirect way by first triggering cytokine release from T-lymphocytes, which then stimulates GTPCH activities in other blood cells. In humans, a biochemical consequence of this immunostimulation is the excretion of both neopterin and 7,8-dihydroneopterin by activated macrophages, and their consequent accumulation in plasma and urine. A physiological function for these compounds has not been established (for a more detailed review of the effects of cytokine stimulation and of the differences between human and murine cells, see Schoedon et al. [23] and Werner et al. [24]). Lipopolysaccharide-treated rats show *de novo* expression and increased enzyme activity of GTPCH in brain, liver, spleen and adrenal gland. In cultured dopamine neurons of the hypothalamus and mesencephalon, cAMP and depolarization of the membrane potential were found to stimulate GTPCH mRNA expression. Increased levels of GTPCH mRNA were also observed in peripheral and central neurons upon treatment with the catecholamine-depleting drug reserpine.

Post-translational processing of GTPCH involves cleavage of the N-terminal 11 amino acids (at least for the rat liver enzyme) and protein phosphorylation [14,25,26,26a]. Whereas a regulatory effect of the N-terminal processing is not known, phosphorylation has been shown to modulate enzyme activity: (1) agents that stimulate protein kinase C, such as phorbol ester, platelet-derived growth factor and angiotensin II, or specific protein kinase C inhibitors, caused an increase or decrease, respectively, of GTPCH phosphorylation; (2) concomitantly, phosphorylation coincides with elevated enzyme activity and an increase in cellular BH_4 levels; and (3) *in vitro* phosphorylation with purified enzymes demonstrated that GTPCH is modified by casein kinase II and/or protein kinase C. The primary amino acid sequence of GTPCH reveals several conserved sites for potential phosphorylation by casein kinase II. However, only one serine residue (Ser-167 in the rat and mouse sequences), which is conserved between the human, rat, mouse and *Drosophila* enzymes and which is exposed at the protein surface, has been proposed to be a potential target site for protein kinase C.

Further effectors of GTPCH enzymic regulation are its substrate GTP, the pathway end-product BH_4, and phenylalanine. The intracellular level of GTP modulates GTPCH activity by cooperative binding and thereby changing the enzyme kinetics. BH_4 and phenylalanine modulate enzymic activity via GFRP, which binds to GTPCH, thereby inducing an as yet unknown conformational change. GFRP mediates end-product feedback inhibition by BH_4. The inactive complex can be converted back into an active form by phenylalanine. The GFRP protein has a monomeric mass of 9.5 kDa and, as shown by cross-linking experiments and sedimentation analysis, is a homopentamer in solution. It was hypothesized that a GFRP pentamer binds to each of the outer faces of two pentamers of GTPCH, as for the binding of GroES to GroEL [27a–27c]. The structure of the protein and the binding sites for the effectors remain to be determined. GFRP mRNA studies by Northern blot analysis and *in situ* hybridization revealed that the expression pattern in rat tissues correlates with that of GTPCH, i.e. GFRP is expressed in peripheral organs such as liver and heart, and also in the brain [27,28]. A physiological consequence of GFRP action is the high plasma BH_4 concentrations observed in patients with hyperphenylalaninaemia caused by phenylalanine 4-hydroxylase (PAH) deficiency.

PTPS

PTPS is considered to be constitutively expressed, but is not ubiquitously present in higher animals (see also below). However, following immunostimulatory induction of GTPCH by cytokines, PTPS can become the rate-limiting enzyme in BH_4 biosynthesis, at least in humans, where PTPS activity is much lower than in rodents [24,29,30]. Whereas GTPCH activity can be stimulated up to 100-fold in cytokine-treated cells, PTPS activity remained unaffected in some experiments, but was stimulated in others. In any case, PTPS activity was reported to be maximally elevated by a factor of 2–4 [29,31–34]. The molecular basis for the stimulation is unclear, i.e. whether it is *de novo* transcription or a post-translational effect. In at least one report, elevation of PTPS mRNA (and GTPCH mRNA; see above) by a factor of 3–4 was observed in rat adrenal glands following treatment with reserpine [35]. A regulatory mechanism for PTPS expression at the mRNA (or pre-mRNA) level was observed for the human myelomonocytic cell line THP-1. Although normal levels of PTPS mRNA in comparison with fibroblasts could be detected by Northern blot analysis, PTPS protein levels and enzymic activity in THP-1 cells were very low

(5% of the activity in fibroblasts). A detailed study of mRNA revealed the presence of two types of PTPS transcripts in THP-1 cells: the normal functional mRNA and a species lacking the 23 bp exon 3, which was eight times more abundant than the wild-type transcript [36]. Furthermore, mRNA species lacking exon 3 have also been detected in human brain cDNA libraries and in a PTPS-deficient patient [37–39]. The significance of such a post-transcriptional event as a potential regulatory mechanism controlling the level of expression of PTPS in other cells remains to be clarified.

With regard to post-translational modifications, PTPS isolated from rat liver was found to be N-terminally processed by the removal of the first four amino acids [40]. Furthermore, human PTPS at least has been shown to be subject to regulatory phosphorylation at Ser-19, whereby cGMP-dependent protein kinase type II seemed to be responsible for the phosphoserine modification [41]. The molecular basis for the at least 3-fold greater activity of the phosphorylated PTPS compared with the non-modified protein when tested in COS-1 cells is not yet understood. Nevertheless, phosphoserine modification appears to be essential, as a phosphorylation-deficient mutant of PTPS was identified from a patient with a defect in BH_4 biosynthesis [42]. It was speculated that, in cultured dopamine neurons, where BH_4 biosynthesis can be stimulated by cAMP, the observed short-term increase in BH_4 levels may be attributed to cAMP-dependent phosphorylation of PTPS [35].

SR

Not much is known about the expression and regulation of mammalian SR. However, there is no indication that the rate of BH_4 biosynthesis is controlled by SR. SR activity remains unaffected by cytokine treatment, and a deficiency of SR in human organs due to genetic defects has not been reported. From N-terminal protein sequence analysis of SR purified from rat erythrocytes, it is known that the protein begins with an *N*-acetylmethionine residue [42a]. Furthermore, SR has been reported to be phosphorylated *in vitro* by calmodulin-dependent protein kinase II and by protein kinase C [43]. Although *in vitro* phosphorylation modified the kinetic properties of the purified rat enzyme, no such modification under cell-culture conditions or in tissue samples has yet been reported.

Localization of biosynthetic enzymes

As shown (at least in rats) by immunohistochemical staining with anti-GTPCH and anti-PTPS antibodies, the expression pattern in various tissues and cell types is highly specific, and co-localization was generally found with aromatic amino acid hydroxylases [44] (A. Resibois and B. Thöny, unpublished work). Unfortunately, no data are available on mapping for the tissue distribution of SR. Interestingly, the immunohistochemical studies on GTPCH and PTPS revealed a nuclear localization for these two BH_4 biosynthetic proteins in specific but various cell types. Subsequent studies were recently performed by transient cell transfections of tagged enzymes for *in situ* immunolocalization by confocal microscopy. In addition to a cytosolic localization, nuclear staining was unequivocally confirmed for GTPCH and PTPS, but not SR, at least in transfected COS-1 and HeLa cells (B. Thöny, A. Resibois and S. Laufs, unpublished work).

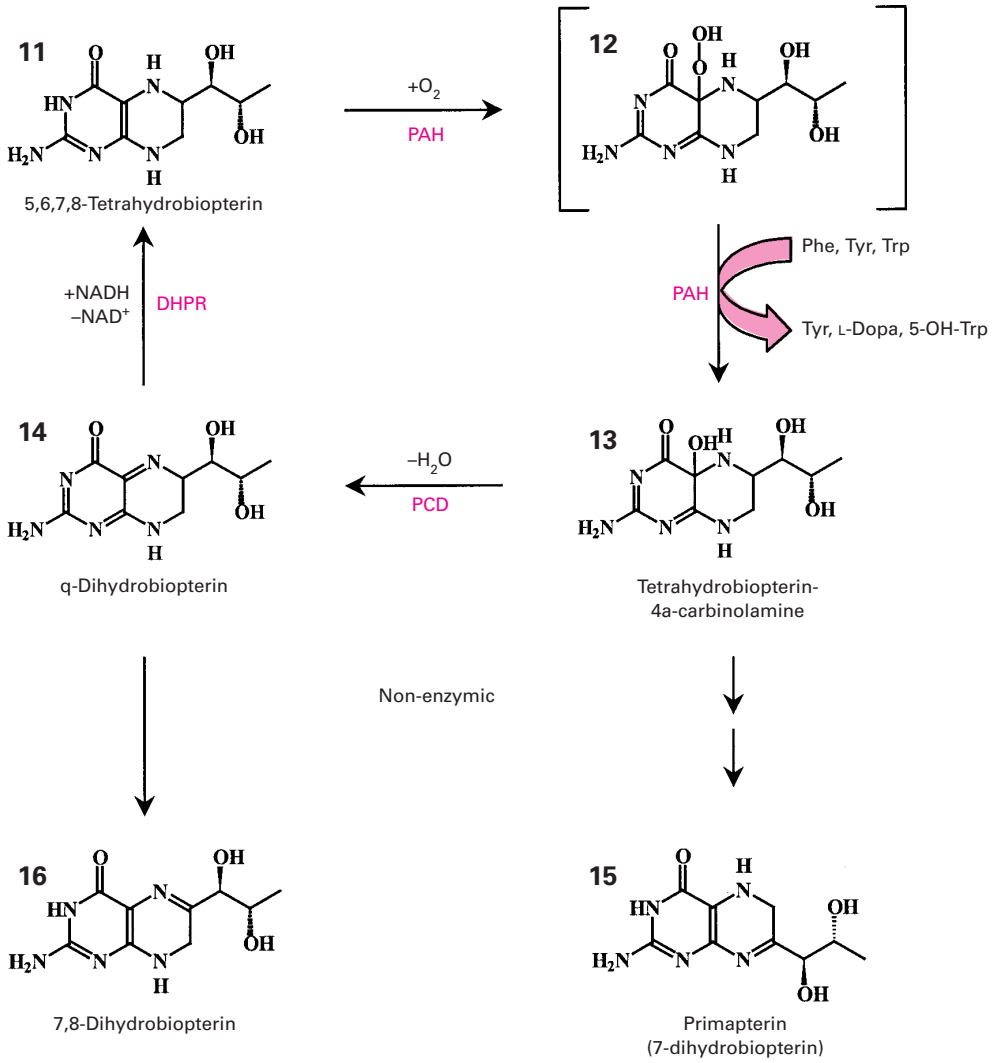
REGENERATION OF BH_4

Regeneration of BH_4 is an essential part of the phenylalanine hydroxylating system (see also 'Cofactor functions' section). During the catalytic event of aromatic amino acid hydroxylases, molecular oxygen is transferred to the corresponding amino acid and BH_4 is oxidized to BH_4-4a-carbinolamine (Scheme 2). Two enzymes are involved in its subsequent dehydratation and reduction to BH_4: pterin-4a-carbinolamine dehydratase (PCD; EC 4.2.1.96) and dihydropteridine reductase (DHPR; EC 1.6.99.7). Enzymic recycling of BH_4 is essential for phenylalanine metabolism: (1) to ensure a continuous supply of reduced cofactor, and (2) to prevent accumulation of harmful metabolites produced by rearrangement of BH_4-4a-carbinolamine. Unexpectedly, the primary structure of PCD is identical with that of a protein of the cell nucleus, named dimerization cofactor (DCoH) of hepatocyte nuclear factor 1α (HNF-1α), reported recently to have a general transcriptional function [45,46]. In the following, PCD will be designated as PCD/DCoH protein.

Reaction mechanism of the regeneration pathway

The dehydratation of BH_4-4a-carbinolamine (compound **13**), the first product of the reaction of aromatic amino acid hydroxylases (Scheme 2), is catalysed by the enzyme PCD/DCoH. The human cytoplasmic PCD/DCoH, the sequence of which is identical with that of the rat protein, is a homotetramer with a molecular mass of 11.9 kDa per subunit [47,48] (Figure 1). Using chemically synthesized pterin-4a-carbinolamine, it has been shown that the enzyme shows little sensitivity to the structure or configuration of the 6-substituent of its substrate, or to the 4a(*R*)-and 4a(*S*)-hydroxy stereoisomers [49]. Obviously, the binding pocket has a relatively high degree of flexibility and might not be designed to recognize only BH_4-4a-carbinolamine. X-ray crystal structures of the tetrameric enzyme complexed with the product analogue 7,8-dihydrobiopterin (compound **16**) revealed four active sites harbouring three essential and conserved histidines (His-61, His-62 and His-79 in the human and rat enzymes; see Figure 3a [50]). Detailed enzymic studies on the stereospecificity and catalytic function revealed a dehydratation mechanism in which the three histidines in PCD/DCoH are crucial for activity [51,52]. A His-61 $\rightarrow$ Ala/His-62 $\rightarrow$ Ala double mutant was fully inactivated and showed a significantly increased dissociation constant with quinonoid 6,6-dimethyl-7,8-dihydropterin. Moreover, His-61 and His-79 act as general acid catalysts for the stereospecific elimination of the 4a(*R*)- and 4a(*S*)-hydroxy groups respectively (Figure 3a). The role of His-62 is primarily to bind substrate, with an additional component of base catalysis [52]. The quinonoid dihydrobiopterin (compound **14**) product is a strong inhibitor of PCD/DCoH, with a K_i value of about one-half of its respective K_m value, and no inhibition was observed with 7,8-dihydrobiopterin (compound **16**) [49]. Furthermore, PAH is not inhibited by its cofactor product, BH_4-4a-carbinolamine, but by primapterin (compound **15**). In the absence of PCD/DCoH, dehydratation of BH_4-4a-carbinolamine also occurs non-enzymically, but at a rate that is, at least in liver, insufficient to maintain BH_4 in the reduced state [53]. As a consequence, liver PCD/DCoH deficiency in humans causes BH_4-4a-carbinolamine to be rearranged via a spiro structure intermediate to dihydroprimapterin (7-substituted dihydrobiopterin; compound **15**) that is excreted in the urine [54,55].

The final conversion of quinonoid dihydrobiopterin into BH_4 is carried out by the dimeric DHPR (Scheme 2). Although the crystallographic structure of the DHPR–NADH binary complex has been solved, the location of the active site is not known from these studies. Nevertheless, an active-site pocket involving the Tyr-Xaa-Xaa-Xaa-Lys motif (Tyr-150 in human DHPR), typical of short-chain dehydrogenases, was proposed to participate in proton donation. Following the classical mechanisms of de-



Scheme 2 Reaction mechanism for the *de novo* pathway for BH_4 regeneration

See the text for details.

hydratation, one molecule of water is released and the product, quinonoid dihydrobiopterin, is reduced back to BH_4 in an NADH-dependent reaction. This final reaction of the regeneration pathway involves direct hydride transfer from the reduced nicotinamide ring to the quinonoid dihydrobiopterin by DHPR. This reaction is supported by the proposed enzyme mechanism of NAD(P)H-dependent reductases and by the lack of detectable prosthetic groups such as flavin or metal ions [56]. The hydride transfer occurs from the B-face of NADH, with transfer of the pro-S hydrogen.

Enzyme structures

PCD/DCoH

The crystal structure of cytoplasmic PCD/DCoH from human and rat liver has been solved [57,58]. The single-domain monomer of 103 amino acids comprises three α-helices packed against one side of a four-stranded, antiparallel β-sheet. The functional enzyme is a homotetramer in which each of the monomers contributes one helix (helix $\alpha 2$) to a central four-helix bundle (Figure 1). In the tetramer, two monomers form an eight-stranded antiparallel β-sheet, with six helices packing against it from one side. The concave, eight-stranded β-sheet with its two protruding loops at either end is reminiscent of the saddle-like shape seen in the TATA-box binding protein. The overall dimensions of the tetramer are 60 Å × 60 Å × 60 Å.

To probe the relationship between dehydratase activity and transcriptional coactivator functions, the X-ray crystal structures of the free enzyme and its complex with the product analogue 7,8-dihydrobiopterin were solved [50]. The ligand binds at four sites per tetrameric enzyme, with little apparent conformational change in the protein. The pterin binds within an arch of aromatic residues that extends across one dimer interface. The bound ligand makes contacts with the three conserved histidines, and this arrangement restricts proposals for the enzymic mechanism of dehydratation. PCD/DCoH binds as a dimer to the helical dimerization domain of HNF-1α. A mutant of PCD/DCoH (Cys-81 → Arg; [59]) with reduced dehydratase activity was not affected in protein–protein interaction and still bound

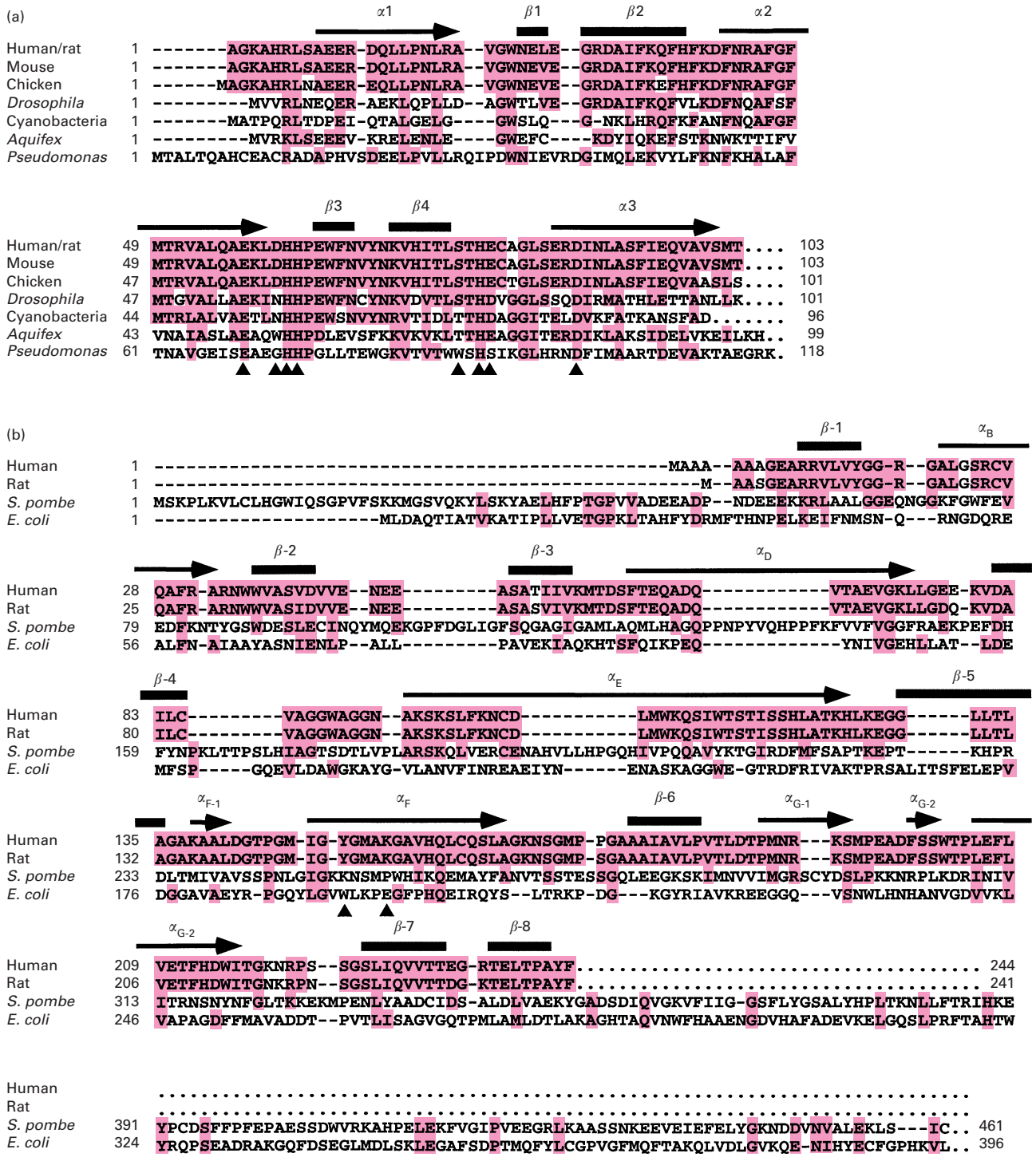


Figure 3 Amino acid sequence alignments of the BH_4-regenerating enzymes (a) PCD/DCoH and (b) DHPR

Alignments were generated using the Clustal W program [141]. Identical residues and residues with functional similarities (V/L/I, R/K, M/L, D/E) are highlighted. Residues in PCD/DCoH involved in catalysis and/or substrate binding are marked with a triangle. For DHPR, the potential active-site-pocket residues are marked with a triangle. The accession numbers for the amino acid sequences for (**a**) PCD/DCoH are P80095/A47010 (human), A47189 (rat), M38741 (mouse), AA06395 (chicken), AAC25196 (*Drosophila*), BAA17842 (cyanobacteria), AAC06420 (*Aquifex*), P43335 (*Pseudomonas aeruginose*); and for (**b**) DHPR are P09417 (human), P11348 (rat), AAA57051 (*Saccharomyces pombe*) and P24232 (*E. coli*).

to HNF-1α, showing that enzymic activity is not essential for HNF1 binding [60]. On the other hand, it was reported that PCD/DCoH retained its enzymic activity while complexed with HNF1 as an $\alpha_2\beta_2$ heterotetramer [61]. Interestingly, the PCD/DCoH homologue in *Pseudomonas aeruginosa phhB* (see below) has dehydratase activity as a dimeric enzyme [62].

The amino acid sequences of the mature human and rat liver proteins are identical, and the mouse and chicken proteins vary

by only one and six amino acids respectively [45,63,64]. Similar proteins have also been found in *Drosophila melanogaster*, and in various bacteria (the Cyanobacterium species *Synechocystis*, *Aquifex aeolicus* and *Pseudomonas aeruginosa*) (Figure 3a).

DHPR

The structures of binary complexes of rat [65] and human [66] DHPRs have been determined by X-ray crystallography (Figure 1). DHPR is an α/β protein, with a central twisted β-sheet flanked on each side by a layer of α-helices. The β-sheet has seven parallel strands and a single antiparallel strand at one edge leading to the C-terminus of the protein. Connections between individual β-strands involve α-helices. Exceptionally, β_2 and β_3 are joined by a short stretch of polypeptide in random-coil conformation. The overall enzyme dimensions are 34 Å × 50 Å × 73 Å. The topology of the backbone folding of DHPR is quite distinct from that of dihydrofolate reductase, although the first six strands of the central β-sheet in DHPR have the same overall topological connectivity as that found for the coenzyme-binding domains of several other $NAD(P)^+$-dependent dehydrogenases. In contrast with the rat enzyme, human DHPR contain two bound NADH molecules per dimer; however, despite the sequential amino acid changes, there are only small differences between the two structures (Figure 3b) [66].

Genes encoding the regenerating enzymes

PCBD

Human PCD/DCoH is encoded by a single-copy gene, *PCBD*, which is located to chromosome 10q22 [67] and composed of four exons [68,69]. Exon 1 is rather short in all known *PCBD* genes from higher eukaryotes, containing only 51, 19 and 23 bp for the hen, rat and human enzymes respectively [64]. Unusually, this first exon codes for a single amino acid only, the starting methionine, which is separated from the subsequent alanine codon by intronic DNA of more than 2 kb length in all three species. The 103-amino-acid active protein from human and rat liver is encoded by exons 2–4. The sequence of the *Drosophila melanogaster* gene, *gpCD1*, revealed that it is interrupted by two introns of 82 and 258 nucleotides [70]. *Pseudomonas aeruginosa*, a Gram-negative bacterium, possesses a multi-gene operon that includes a gene (*PhhB*) encoding a homologue of the regulatory PCD/DCoH, together with genes encoding PAH (*PhhA*) and aromatic aminotransferase (*PhhC*) [71].

Within the human *PCBD* 5′ flanking sequence, potential regulatory regions include consensus binding sites for transcription factor Sp1, an AP-1 (activator protein-1) and several AP-2 binding sites; however, the 5′ upstream region lacks both proximal TATA- and CAAT-box promoter elements [69]. In addition, a comparison of the putative promotor regions between the human, rat and chicken *PCBD* genes revealed that all three promoters are located within a region of increased GC content (hen 64%, human 64%, rat 56%) [64]. Whether *PCBD* is transcribed at a basal level by housekeeping factors and further modulated by additional transcriptional elements has yet to be determined.

QDPR

The human *QDPR* gene is located on chromosome 4p15.3. It extends over more than 20 kb, and the coding sequence consists of 732 bp contained in seven exons ranging in size from 84 to 564 bp. Unfortunately, nothing is yet known about the *QDPR* promoter, except that it appears to be GC-rich in sequence [72].

Regulation of enzyme expression and activity

PCD/DCoH

PCD/DCoH was originally detected as a contaminant in a preparation of rat PAH as a consequence of its ability to stimulate the BH_4-dependent hydroxylation of phenylalanine [47]. This stimulatory protein was subsequently purified from rat liver [73] and its activity was shown to be due to the catalysis of dehydratation of the 4a-carbinolamine intermediate. PCD/DCoH activity has also been shown to be present in human liver [63], kidney and brain [74], skin [75,76] and hair follicles [77]; its absence is concomitant with the formation of 7-substituted pterins (see above). PCD/DCoH activity, as a rule, is low in those tissues that contain high levels of tyrosine and tryptophan hydroxylase activity, except for the pineal gland. On analysing tissues from adult rats using PCD/DCoH-specific antibodies in Western blots, the protein was localized in liver and kidney, and in smaller amounts in stomach and intestine [78]. PCD/DCoH is therefore, together with HNF-1α, found in liver and kidney, organs known to express these transcription factors. In liver, all the hepatocytes, but not the other cell types, are immunoreactive [79]. In kidney, the protein is prevalent in the proximal and distal convoluted tubules; in the adrenal gland all the cells of the medulla are labelled; in brain, it generally co-localizes with tyrosine hydroxylase. Positive nerve cells occur in myenteric ganglia of the whole gastrointestinal tract and in the intestinal submucosal ganglia. The prominent nuclear immunoreactivity found in all neural crest, but also in other cell types that do not express either HNF-1α or aromatic amino acid hydrolases, argues in favour of a novel, as yet unknown, function of the protein [79,80].

No clear link has so far been established between the enzyme activity of PCD/DCoH and its ability to form stable hetero-tetramers with HNF-1α. Although PCD/DCoH stabilizes HNF-1α, the enzymic activity *per se* is not essential for HNF-1α binding. Using a yeast two-hybrid system it has been shown that naturally occurring substitution mutants of PCD/DCoH with impaired enzymic activity still bind to HNF-1α *ex vivo* [60]. Thus binding to HNF-1α does not interfere with the integrity of the active site of PCD/DCoH.

In mice lacking HNF-1α, the rate of transcription of genes such as those encoding albumin and α1-antitrypsin is reduced, while the gene coding for PAH is totally silent, giving rise to phenylketonuria [81]. Mutant mice also suffer from severe Fanconi syndrome caused by renal proximal tubular dysfunction. In order to prove that PCD/DCoH could enhance the expression of PAH, a number of co-transfection studies were carried out [82]. PCD/DCoH itself could not transactivate the 9 kb human PAH 5′ flanking fragment; however, it was transactivated by HNF-1α in a dose-dependent manner with a maximum of nearly 8-fold activation, and PCD/DCoH potentiated this trans-activation by another 1.6-fold. These data suggest that the dehydratase can enhance the expression of the human PAH gene.

In *P. aeruginosa*, *PhhB*, the homologue of PCD/DCoH, is required for *in vivo* function of phenylalanine hydroxylase (*PhhA*). The *PhhB* requirement can be substituted by its mammalian PCD/DCoH counterpart [71].

DHPR

The relatively high levels of DHPR, compared with those of aromatic amino acid hydroxylases, and its presence in tissues lacking these enzymes imply that DHPR may be involved in other metabolic processes. For example, there is evidence that DHPR in the presence of NADH could preserve tetrahydrofolate

levels in brain where the concentrations of dihydrofolate reductase are low [83]. DHPR is widely distributed in animal tissue [84]. Its occurrence in brain and adrenal medulla is not surprising in view of its role in the tyrosine hydroxylation system in these tissues, and in tryptophan hydroxylation in brain. However, why DHPR should be found in tissues such as heart and lung, which have little or no aromatic amino acid hydroxylating activity, is obscure. DHPR activity has also been detected in cultured fibroblasts, amniocytes, lymphocytes, erythrocytes and platelets.

Detailed studies by immunoprecipitation and two-dimensional electrophoresis have shown that DHPRs from liver, Epstein–Barr-virus-transformed lymphoblasts and fibroblasts are identical [85].

FUNCTIONS OF BH_4

Cofactor functions

One of the best investigated functions of BH_4 is its action as a natural cofactor of the aromatic amino acid hydroxylases, i.e. PAH (EC 1.14.16.2), tyrosine 3-hydroxylase (EC 1.14.16.3) and tryptophan 5-hydroxylase (EC 1.14.16.4), as well as of all three forms of nitric oxide synthase (NOS). In addition, BH_4 is required by the enzyme glyceryl-ether mono-oxygenase (EC 1.14.16.5) for hydroxylation of the α-carbon atom of the lipid carbon chain of glyceryl ether to form α-hydroxyalkyl glycerol [86]. The significance of glyceryl-ether mono-oxygenase in humans has been well documented; however, there is so far no information about the consequences of BH_4 deficiency on alkyl ether metabolism.

The enzymic reactions of aromatic amino acid hydroxylases have been intensively studied by Kaufman and others, who showed that they have many features in common [87–90]. They all have a strict requirement for oxygen, iron and BH_4, and the oxidation product of BH_4 is regenerated by the enzymes PCD/DCoH and DHPR (Scheme 2). The oxidation of BH_4 involves the formation of the BH_4-4a-carbinolamine intermediate (compound **13**), and this has been shown to be formed in the reactions of both PAH [48,91] and tyrosine hydroxylase [92,93]. Studies on PAH found that a stoichiometric amount of BH_4 can be oxidized in the presence of oxygen, and this yields the reduced enzyme. It has been proposed that this reductive activation of PAH occurs at the redox site and that the enzyme's iron is a part of this redox. Its reduction from Fe^{3+} to Fe^{2+} has been linked to the formation of active PAH. Two electrons from BH_4 are required to reduce the enzyme; one is transferred to Fe^{3+} and the second apparently to oxygen [94]. The function of BH_4 as a cofactor for NOS is different (dimer stabilization), and BH_4 seems not to be regenerated during the catalytic event.

Phenylalanine and BH_4 are the major regulators of PAH [94]. While phenylalanine is a positive allosteric effector (activator) that converts inactive enzyme into catalytically competent (activated) enzyme, BH_4 is a negative effector that competes with phenylalanine activation to form a dead-end complex (PAH–BH_4) [95]. Thus BH_4 plays a central regulatory role in the phenylalanine hydroxylating system. The only other known BH_4-requiring enzymes in liver, glyceryl-ether mono-oxygenase and NOS, are present in relatively low amounts, and PAH (subunit) and BH_4 concentrations in liver are approximately equal (8–9 μM) [96]. As a consequence, formation of the PAH–BH_4 complex will cause equal decreases in free enzyme and free BH_4 concentrations, and phenylalanine, by controlling the activation of PAH, will control both the metabolic availability of BH_4 and the amount of active PAH in a cell [97]. There is no evidence that tyrosine hydroxylase or tryptophan hydroxylase is regulated by substrate-activated mechanisms similar to those that regulate PAH. All three aromatic amino acid hydroxylases are inhibited by catecholamines, but only the inhibition of human tyrosine hydroxylase is competitive with respect to the BH_4 cofactor, and it has been shown that the cofactor can directly displace dopamine from the enzyme active site [98].

It has been shown that BH_4 stimulates all NOS isoforms [inducible (iNOS), neural (nNOS) and endothelial (eNOS)] [99–101], and basal enzyme activity is due to a residual amount of BH_4 bound tightly to protein. The correlation of the amount of bound BH_4 with enzyme activity clearly suggested that it is an essential cofactor of NOS; however, its exact function in the conversion of arginine into citrulline is still unclear [102]. By analogy with its function in aromatic amino acid hydroxylases, it has been suggested that BH_4 can serve as electron donor in the NOS reaction [103]; however, direct involvement in neither substrate oxidation nor its regeneration could be demonstrated [104]. In contrast, it has been demonstrated that the reduction of quinonoid dihydrobiopterin (qBH_2) to BH_4 is catalysed by NOS through its flavoprotein 'diaphorase' activity [105]. BH_4 seems not to be catalytically active, but represents an allosteric effector of the enzyme [101]. Recently, Raman et al. [106] reported the crystal structure of constitutive eNOS in BH_4-free and bound forms. The observed data suggest that a specific recognition of L-arginine at the BH_4 site in eNOS stabilizes a positively charged state of the pterin ring (BH_4^-) and, particularly, the pterin cation radical ($BH_4^{+\bullet}$). The uniqueness of the BH_4–eNOS interaction via the hydrogen-bond network and the ability to bind L-arginine at the pterin-binding site present strong arguments for the involvement of a pterin radical in NOS catalysis, and rule out the possibility of $BH_4 \leftrightarrow qBH_2$ recyling during NO biosynthesis [106].

The requirement for the BH_4 cofactor is much lower for the NOS enzyme than for PAH. The K_m values for BH_4 for PAH and NOS are 2–3 μM and 0.02–0.03 μM respectively. Pastor et al. [107] questioned the importance of competition of BH_4 between these two hepatic enzymes. They showed that basal BH_4 synthesis appears to be adequate to support iNOS activity, whereas BH_4 is increased to support PAH activity. Phenylalanine markedly increased BH_4 biosynthesis (via GFRP; see above), whereas arginine had no effect. The $K_m(BH_4)$ values for the two brain enzymes tyrosine hydroxylase and tryptophan hydroxylase are ~ 30 μM.

Cellular functions

One of the earliest cellular functions of BH_4 to be discovered was as a growth factor for *Crithidia fasciculata*, and this was initially used to measure biopterins in different body fluids and tissues. More recent observations suggested proliferative activity of BH_4 in haemopoietic cells [108]. Exogenous BH_4 was found to stimulate DNA synthesis and induce proliferation of some mouse erythroleukaemia clonal cell lines [108,109]. BH_4 and sepiapterin also enhanced the proliferation of simian virus 40 (SV40)-transformed human fibroblasts and rat C6 glioma cells, indicating that the stimulatory effect of BH_4 on cell proliferation is not restricted to PC12 cells. Subsequently it has been shown that epidermal growth factor and nerve growth factor increased the proliferation of rat PC12 cells through obligatory elevation of intracellular BH_4 [110].

Besides its proliferative activity, BH_4 has also been suggested to act as a self-protecting factor for NO toxicity, with generation of superoxide in NO-producing neurons [111]. Indeed, strong scavenging activity of BH_4 for superoxide anion radicals was shown with both xanthine/xanthine oxidase and rat macrophage/phorbol myristate acetate radical-generating systems

[112], and the authors suggested that BH_4 might be useful in the treatment of various diseases whose pathogenesis is actively oxygen-related. In another series of experiments, Shimizu et al. [113] demonstrated that *S*-nitroso-*N*-acetyl-D,L-penicillamine (NO donor)-induced endothelial cell death can be prevented by increasing the cellular levels of BH_4. This finding suggested further that the cytotoxicity of NO involves H_2O_2 production, and that scavenging of H_2O_2 by BH_4 may be at least one of the mechanisms by which BH_4 decreases NO-induced endothelial cell death.

Cho et al. [114] suggested another role for BH_4. They hypothesized that ischaemia increases intracellular BH_4 levels, and that the increased BH_4 level plays a critical role in selective neuronal injury via NOS activation. Using a selective inhibitor of GTPCH in animals exposed to transient forebrain ischaemia, they demonstrated a marked decrease in BH_4 levels, NADPH-diaphorase activity and caspase-3 gene expression in the CA1 hippocampus. Moreover, delayed neuronal injury in the CA1 hippocampal region was significantly attenuated by the GTPCH inhibitor. These data, in contrast with those of Shimizu et al. [113], suggested that a blockade of BH_4 biosynthesis may provide novel strategies for neuroprotection.

BH_4 in disease

BH_4 deficiency is associated with a rare variant of hyperphenylalaninaemia ('atypical' or 'malignant' phenylketonuria) that is unresponsive to a low-phenylalanine diet [115]. Phenotypically it presents with a deficit of the neurotransmitters dopamine and 5-hydroxytryptamine (serotonin) and progressive neurological symptoms [116]. It is a heterogeneous group of diseases affecting either all organs, including the central nervous system, or only the peripheral hepatic phenylalanine hydroxylating system [117, 118]. BH_4 deficiency can be caused by mutations in genes encoding the enzymes involved in its biosynthesis [39] (GTPCH and PTPS) or regeneration [72,119–121] (PCD/DCOH and DHPR). The mutations are all inherited in an autosomal recessive manner. Biochemical, clinical and DNA data from patients with BH_4 deficiencies are tabulated in the BIODEF and BIOMDB databases, and are available on the Internet (www.unizh.ch/~blau/bh4.html) [122]. Two forms of BH_4 deficiency may occur without hyperphenylalaninaemia. The autosomal dominantly inherited and compound heterozygote form of GTPCH deficiency (Dopa-responsive dystonia, initially described as Segawa disease [123]), together with an apparent central nervous system-localized form of DHPR deficiency [124], have recently been recognized, none of which have been associated with elevated plasma phenylalanine in infancy. (For a more detailed review of BH_4 deficiencies, see Blau et al. [125].)

Decreased levels of BH_4 in the cerebrospinal fluid have also been documented in other neurological diseases presenting phenotypically without hyperphenylalaninaemia, such as Parkinson's disease [126], autism [127], depression [128] and Alzheimer's disease [129]. In some of these, administration of BH_4 has been reported to improve the clinical symptoms [126, 130,131]. Unfortunately, others were not able to confirm these results [132], and thus a benefit of BH_4 therapy, at least for this group of diseases, is questionable.

Neurotransmitter-releasing function

It has been shown that BH_4 enhances the release of dopamine [133] and 5-hydroxytryptamine [134] in the rat striatum when administered locally through the dialysis membrane. The enhancement of dopamine release persisted even when dopamine biosynthesis or dopamine re-uptake was completely blocked, but it was abolished when blockers of voltage-dependent Na^+ or Ca^{2+} channels were administered along with BH_4. Further experiments using selective inhibitors of tyrosine, tyrosine hydroxylase and NOS demonstrated that BH_4 stimulates dopamine release directly, independent of its cofactor action on tyrosine hydroxylase and NOS, by acting from the outside of neurons [135]. The exact mechanism is not entirely clear, but it has been shown that arginine also induces a concentration-dependent increase in dopamine release in the superfusate of rat striatum slices, and that this is dependent on the presence of BH_4 [136].

PERSPECTIVE AND OUTLOOK

Molecular analyses spanning the spectrum from gene cloning to protein X-ray structure determinations of all BH_4 metabolic enzymes provide the basis for experimental approaches to understanding BH_4 function. From a medical point of view, a major impact on such comprehension would be the transition from given genetic defects to understanding of the disease mechanism. Yet, for an integrated picture of understanding the molecular biology of BH_4, many questions remain to be answered, including the following. (1) What regulates the specific expression pattern, and what are the molecular consequences of enzyme modifications, including phosphorylation? Is there a role for phosphatases in enzyme dephosphorylation and deactivation? (2) How do GTPCH and GFRP proteins interact, and are there other protein–protein interactions, including a potential biosynthetic 'super complex' of GTPCH, PTPS and/or SR? (3) What is the function of the central pores in GTPCH and PTPS? (4) Why are at least three out of the five metabolic proteins also present in the nucleus? (5) Why are some human GTPCH mutations dominant, leading to Segawa syndrome, and what is the mechanism of peripheral compared with central types of PTPS or DHPR deficiencies? Furthermore, why are there no patients with SR deficiency?

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