REVIEW ARTICLE

Structure and function of the aromatic amino acid hydroxylases

Simon E. HUFTON, Ian G. JENNINGS and Richard G. H. COTTON*

Olive Miller Protein Chemistry Laboratory, Murdoch Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia

INTRODUCTION

The three aromatic amino acid hydroxylases, phenylalanine hydroxylase (PheOH; EC 1.14.16.1), tyrosine hydroxylase (TyrOH; EC 1.14.16.2) and tryptophan hydroxylase (TrpOH; EC 1.14.16.4), constitute a family of enzymes that share many physical, structural and catalytic properties. PheOH catalyses the hydroxylation of phenylalanine to tyrosine and is the rate-limiting step in the only pathway to catabolize phenylalanine (for reviews see [1,2]). TyrOH catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-Dopa), which is the rate-limiting step in the biosynthesis of catecholamines (for reviews see [3,4]). TrpOH is the rate-limiting step in the biosynthesis of 5-hydroxytryptamine (serotonin), and catalyses the hydroxylation of tryptophan to 5-hydroxytryptophan (for reviews see [5,6]). The general characteristics of these enzymes have been most commonly established through studies on hepatic PheOH and extended to include TyrOH and TrpOH. Although the majority of information concerning the structure and function of aromatic amino acid hydroxylases relates to PheOH, this review attempts to discuss these enzymes as a group that share many properties.

The hydroxylation of aromatic amino acids requires a non-protein cofactor which was shown to be tetrahydrobiopterin (BH_4) based on its ability to promote the hydroxylation of phenylalanine to tyrosine. The active cofactor is the reduced tetrahydrobiopterin, and it is the enzyme dihydropteridine reductase which catalyses its regeneration by the reduction of the dihydrobiopterin back to BH_4 (Figure 1). The establishment of an essential role for BH_4 and dihydropteridine reductase in the PheOH reaction was subsequently extended to include the TyrOH and TrpOH reactions.

Before examining in detail the hydroxylase enzyme systems, it is important to place the amino acids involved in the context of mammalian metabolism. The conversion of phenylalanine into tyrosine serves two purposes. First, it provides an endogenous supply of tyrosine, making tyrosine a non-essential dietary component. Secondly it is the rate-limiting step in the only metabolic pathway by which phenylalanine can be completely catabolized to carbon dioxide and water. In phenylketonuria (PKU) patients the enzyme PheOH is defective, and this results in an elevation of serum levels of phenylalanine called hyperphenylalaninaemia (for a review see [7]). This metabolic block prevents the complete combustion of phenylalanine and prevents the breakdown of the benzene ring, resulting in phenylketones being excreted in the urine. Another consequence of classical PKU is that tyrosine becomes an essential dietary component required for the synthesis of the neurotransmitter dopamine. Excess phenylalanine and its metabolites in serum cause brain damage without dietary management, which is the recognized treatment for this disorder. Non-responsiveness to dietary treatment of PKU has led to the identification of other hyperphenylalaninaemias resulting from defective genes other than that for PheOH. These other genes code for enzymes which relate to the production of the cofactor BH_4 and its recycling.

The hyperphenylalaninaemias are caused by a defect either in PheOH or in the production and recycling of BH_4. The former is diagnosed as PKU or benign hyperphenylalaninaemia, based purely on the levels of phenylalanine in the blood. The latter, so-called malignant hyperphenylalaninaemia or BH_4 deficiency, is caused by defects in three enzymes, dihydropteridine reductase, 6-pyruvyltetrahydrobiopterin synthase or GTP cyclohydrolase (Figure 1). These enzyme defects in the production of BH_4 have a more severe clinical phenotype because all three aromatic amino acid hydroxylases that exist in mammals are specifically affected. Both TyrOH and TrpOH are required for the synthesis of neurotransmitters and, as such, deficiencies in BH_4 synthesis result in severe neurological symptoms. Actual defects in TyrOH and TrpOH are much more difficult to characterize. Lower activities of these enzymes or lower levels of catecholamines (i.e. dopamine or 5-hydroxytryptamine) have been reported in several disease states, including depression, hypertension and schizophrenia [8–11].

LOCALIZATION AND PURIFICATION OF HYDROXYLASES

The catabolism of phenylalanine in mammals is mainly associated with the liver [1,2], with some activity associated with the kidney tubules in the rat [12–15]. The enzyme PheOH has been localized to hepatocytes using immunohistochemical methods and appears to be distributed throughout the cytoplasm [16]. PheOH has been purified mainly from rat liver [17–20], human liver [21,22], monkey liver [23] and rat kidney [24] and shown to have a subunit molecular mass of 50 kDa. The purification of PheOH had been a laborious process until Shiman introduced a method based on hydrophobic interaction chromatography. A substrate-induced conformational change in PheOH results in an increase in hydrophobicity, and this affords a purification strategy [20].

Both TyrOH and TrpOH are involved in neurotransmitter synthesis and could be expected to be found in tissues of the nervous system. TyrOH has been localized to the adrenal medulla and the catecholinergic neurons of the central or peripheral nervous systems [25]. A number of studies have suggested that TyrOH is found in both a cytoplasmic and a membrane form [26]. The relevance, if any, of this has yet to be determined, but it is interesting to postulate some connection with the activation of TyrOH by phospholipid (see 'Methods of *in vitro* Activation' section). Attempts at purification of TyrOH from a variety of tissues have met with varied success, often producing poor yields and purity. However, significant amounts of active soluble TyrOH have been purified from bovine adrenal medulla and rat pheochromacytoma cells. This enzyme has been shown to have a subunit molecular mass of 60 kDa by SDS/PAGE [27,28]. Expression of purified recombinant TyrOH in both insect tissue

Abbreviations used: PheOH, phenylalanine hydroxylase; TyrOH, tyrosine hydroxylase; TrpOH, tryptophan hydroxylase; BH_4, tetrahydrobiopterin; PKU, phenylketonuria; ANBADP, 2,6-diamino-5-[(3-azido-6-nitrobenzylidene)amino]pyrimidin-4-one; PKA, cyclic AMP-dependent protein kinase; CAM-PKII, Ca^{2+}/calmodulin-dependent protein kinase; ERK, extracellular signal-related protein kinase.

*To whom correspondence should be addressed.

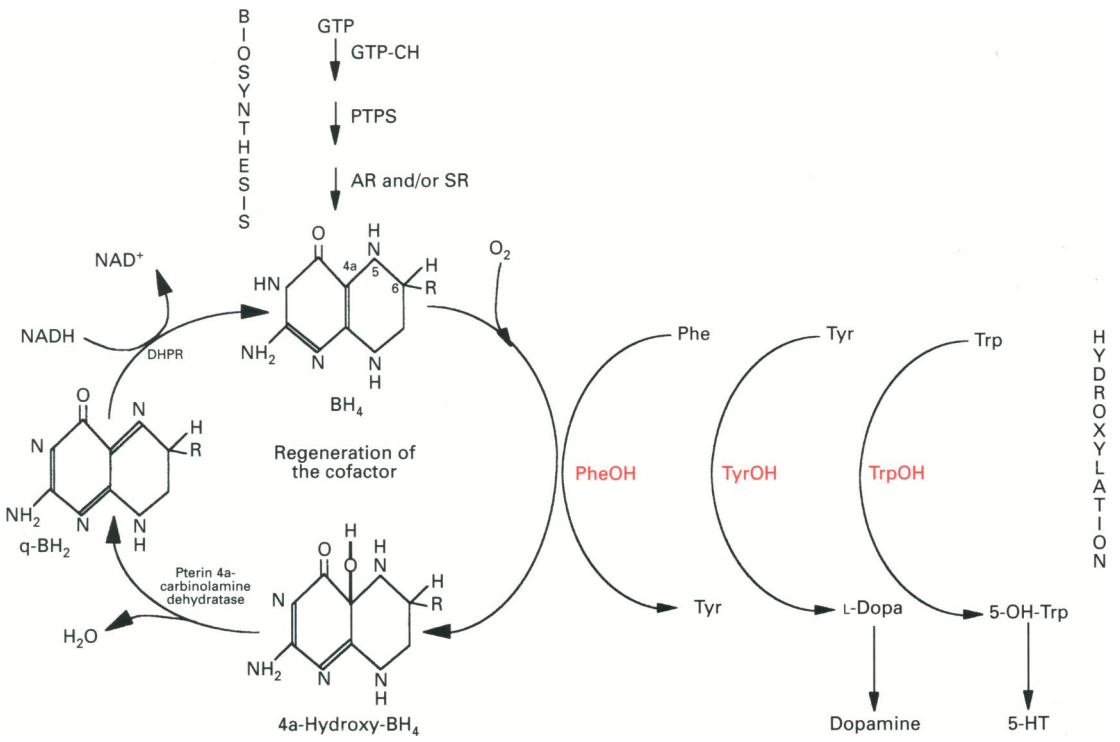

Figure 1 Enzymes involved in the BH_4-dependent hydroxylation of aromatic amino acids

The metabolic pathways connected to the hydroxylation of aromatic amino acids are indicated. GTP-CH (GTP-cyclohydrolase), PTPS (6-pyruvyltetrahydrobiopterin synthase), AR (aldose reductase) and/or SR (sepiapterin reductase) are all involved in the biosynthesis of BH_4 from GTP. Pterin 4a-carbinolamine dehydratase and DHPR (dihydropteridine reductase) are involved in the regeneration of BH_4 and act upon the substrates 4a-hydroxy-BH_4 (or 4a-carbinolamine) and q-BH_2 (quinonoid dihydrobiopterin) respectively. PheOH, TyrOH and TrpOH catalyse the hydroxylation of phenylalanine, tyrosine and tryptophan to tyrosine, 3,4-dihydroxyphenylalanine (L-Dopa) and 5′-hydroxytryptophan respectively. 5-HT, 5-hydroxytryptamine (serotonin).

Table 1 Some biochemical properties of the aromatic amino acid hydroxylases

See the text for references and further details. The subunit molecular mass was calculated from the cDNA sequence. The iron content is given in mol/mol of subunit.

	Location	Subunit molecular mass (Da)	State of multimerization	Isoenzymes	Iron content (mol/mol)	K_m (μM) for:	
						BH_4	Substrate
PheOH	Liver/kidney	51672	(Homodimer)/ homotetramer	Not known	1	15–25	200–300
TyrOH	Adrenal medulla; catecholinergic neurons of peripheral and central nervous systems	55903–59158	Homotetramer	HTH1, HTH2, HTH3, HTH4	1	13–20	6–11
TrpOH	Serotonergic neurons of the central nervous system	51007	Homotetramer	Not known	Contains iron	20–30	12.5–32

culture cells [29] and *Escherichia coli* [30] has been reported, and this represents a significant source of the enzyme.

TrpOH activity has been detected in the brain, the pineal gland and also in the enteric neurons of the gut [31–33]. The subcellular distribution of TrpOH varies from tissue to tissue between a soluble and a particulate form [34]. TrpOH has been purified from a variety of tissues but recovery is generally low [35–37]. Estimation of the molecular mass of TrpOH from a number of tissues has given variable results. The oligomeric form of TrpOH purified from rabbit hindbrain has an estimated molecular mass of 230 kDa [36], composed of identical subunits of estimated molecular mass 58 kDa [34,38]. Table 1 gives a summary of some of the relevant biochemical properties of this family of hydroxylases.

MOLECULAR GENETICS OF HYDROXYLASE GENES

Much effort has been devoted to the study of PKU, and the characterization of this disorder at the molecular level began with the isolation of the full-length human PheOH cDNA [39]. The human PheOH gene was shown to be the product of a single genetic locus by expression of the full-length cDNA in cultured

mammalian cells driven by an appropriate promoter [40] (this step was imperative as it demonstrated that the human cDNA could be used for diagnosis of PKU). With this achievement followed the identification of the first mutant allele by DiLella et al. [41]. Using specific oligonucleotide probes a splicing mutation in the donor site of intron 12 was detected. To date there have been over 200 different mutations identified in the PheOH gene but these have yielded very little structure/function information. The identification of such a large number of separate mutations responsible for PKU indicates that this disease has arisen independently throughout the world as opposed to from a single focus (for a review see Eisensmith and Woo [7,42]).

PheOH cDNA clones from rat [13], mouse [43] and *Drosophila melanogaster* [44] have also been characterized and as expected shown to have a very high degree of identity at both the nucleotide and the amino acid levels to the human form. The human PheOH locus was shown to lie on chromosome 12 and was further localized to the 12q22-q24.1 region [45]. Using the human PheOH cDNA as a probe the genomic sequence of PheOH was shown to span approximately 90 kb with 13 exons and 12 large introns [46].

The genes for TyrOH and TrpOH have been cloned and sequenced from a large number of species and clearly show extensive identity with that of PheOH (TyrOH from rat [47], human [48], bovine [49] and quail [50]; TrpOH from rabbit [51], rat [52], human [53] and mouse [54]). The human TyrOH has been localized to chromosome 11p15-13 [55,56] and is physically located 2.7 kb 5′ to the insulin gene [57]. The TrpOH gene has been localized to chromosome region 11p15-3-p14 [58], and this places the TrpOH gene proximal to the TyrOH locus.

MECHANISM OF HYDROXYLATION

Despite much effort little is known, with certainty, about the mechanism of these enzymes, particularly concerning the nature of the hydroxylating species. The aromatic amino acid hydroxylases require reduced pterin cofactor, molecular oxygen and iron in order to hydroxylate their substrate. The oxidation of BH_4 involves the formation of a 4a-carbinolamine (or 4a-hydroxy-BH_4) intermediate, and this has been shown to be formed in the reactions of both PheOH [59,60] and TyrOH [61,62] (Figure 1). The hydroxy group of 4a-hydroxy-BH_4 appears to come directly from the atom of oxygen that is not incorporated into tyrosine [63,64].

The early discovery that PheOH was an iron-containing enzyme [65] was followed by intensive study, but the detailed role of its metal centre in enzyme mechanism and regulation has remained elusive. Studies on PheOH by Marota and Shiman [66] found that a stoichiometric amount of BH_4 can be oxidized in the presence of oxygen and this yields reduced enzyme. This reductive activation of PheOH by BH_4 has been proposed to occur at a specific redox site, distinct from either the regulatory or catalytic site. The enzyme's iron is part of this redox site [67] and its reduction from Fe^{3+} to Fe^{2+} has been linked to the formation of active PheOH [68]. Two electrons from BH_4 are required to reduce the enzyme; one electron is transferred to Fe^{3+} and the second, apparently, to oxygen [66,68–70]. It has been proposed that Fe^{2+} binds dioxygen [71] and furthermore that a highly reactive enzyme-bound iron–oxo compound is the hydroxylating species [63,64]. Recent results probing the catalytic site of TyrOH suggest a role for Fe^{2+} in mediating the involvement of oxygen in the hydroxylation reaction. Proton NMR was used to determine the conformation of phenylalanine and its proximity to the bivalent cation at the active site of human tyrosine hydrolase [72]. The molecular distances reported between the aromatic ring and the metal ion were consistent with an Fe^{2+}-bound oxy or peroxy species acting as the hydroxylation intermediate. Although several hydroxylating species have been proposed, the precise mechanism of oxygen activation is unknown. As stated earlier, 4a-hydroxy-BH_4 has been detected in both the phenylalanine and tyrosine hydroxylating systems, and it is possible that this could be formed from a 4a-peroxytetrahydropterin species in the oxygen-activation step of TyrOH and PheOH [61,62,73,74]. A speculative model for the active site of PheOH is given in Figure 2.

A kinetic mechanism for the hydroxylation of tyrosine has been proposed by Fitzpatrick [75] in which BH_4, oxygen and tyrosine bind in an ordered fashion. It is believed that a

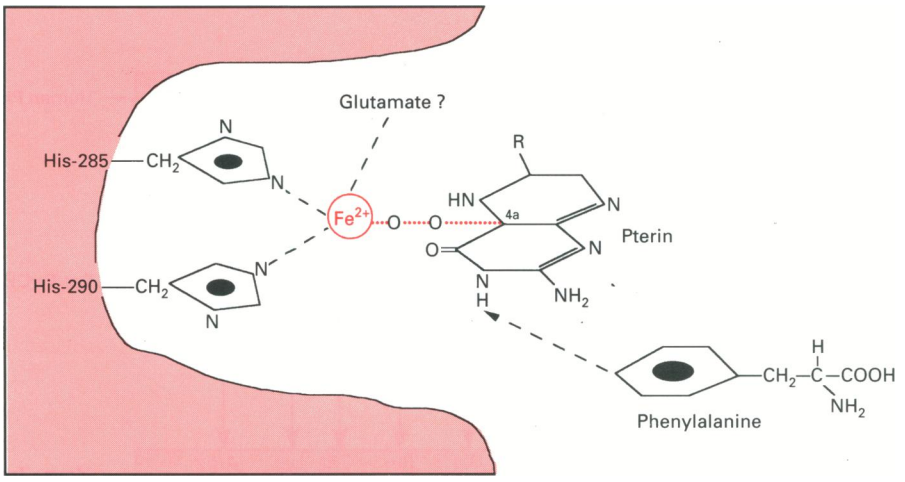

Figure 2 A possible model for the active site of PheOH

The reduced Fe^{2+} at the active site is shown co-ordinately bound to the two imidazole groups of His-285 and His-290, which are reported to be involved in iron binding [113]. The histidine residues correspond to those in rat PheOH and are numbered so as to include the initiator methionine. Further binding of iron by the carboxy group of a glutamate residue is also suggested [105]. BH_4 is proposed to be bound at the active site through an Fe^{2+} peroxy-4a-pterin structure. The substrate phenylalanine is also presented in the model but there are very few data on its function within the active site.

quaternary complex of enzyme·$Fe^{2+}BH_4$·O_2·Tyr is formed before any reversible steps occur. However, the rate-limiting step in catalysis has yet to be unequivocally determined, but results so far suggest that the formation of the hydroxylating BH_4 intermediate is the slow step [75–77]. (For reviews of the mechanism of hydroxylation in PheOH, see [73,78].)

STRUCTURE/FUNCTION CORRELATIONS

Domain structure and homology

Through the comparison of primary sequences of equivalent proteins from different organisms it is often possible to predict functionally important regions. Recent studies have shown that the three BH_4-dependent aromatic amino acid hydroxylases are highly homologous, reflecting a mechanism of evolution from a single primordial locus [79,51]. The highest degree of sequence similarity is restricted to the C-terminal two-thirds of these molecules, possibly representing a conserved catalytic domain, whereas the less similar N-terminal domains probably reflect the unique properties of each enzyme. It is apparent that these enzymes have a domain structure, and this will be discussed later with reference to the catalytic activity, substrate specificity and regulatory mechanisms. The C-terminal domain is generally hydrophobic whereas the regulatory N-terminal domain is generally hydrophilic and includes the phosphorylation sites [43]. Cysteine residues within the aromatic amino acid hydroxylases are significantly biased towards the catalytic domain, with five being absolutely conserved. This is suggestive of a common three-dimensional conformation for the catalytic domain and a less structured non-conserved regulatory domain. The identification of a conserved epitope amongst the three aromatic amino acid hydroxylases again supports the notion of a common structure among this family of enzymes. The epitope for an antibody, PH8, has been localized to residues 139–155 in PheOH (all numbering of residues is taken to include the initiator methionine) which is a highly conserved region within this family of enzymes [80,81]; this region does not appear to be important for enzyme function but may have an important structural role.

To date a large number of hydroxylases have been sequenced from a variety of organisms, and all show extensive identity in the catalytic domain. Two bacterial PheOH genes that have been characterized from *Pseudomonas aeroginosa* and *Chromobacterium violaceum* do not possess the proposed regulatory domain. This is not surprising as the regulatory functions provided in higher eukaryotes by this domain (i.e phosphorylation and phenylalanine activation in the case of PheOH) are not known to exist for bacterial enzymes [82,83].

The evolutionary relatedness of the three aromatic amino acid hydroxylases is of interest from a structure/function point of view. In invertebrates (i.e. *Drosophila melanogaster*) it has been suggested that only two aromatic amino acid hydroxylase genes exist, one gene encoding TyrOH and the other encoding a protein with both TrpOH and PheOH activities [84]. Neckameyer and White [84] suggest the existence of an ancestral hydroxylase gene the product of which most strongly resembles PheOH in structure and function, from which the other hydroxylases developed in line with the evolution of vertebrate nervous systems. This information provides support for a model of duplication from a single locus, with the specificities of TrpOH and PheOH evolving after the initial duplication event. Further evidence for a common evolutionary origin amongst the hydroxylases is seen when the positions of the intron/exon boundaries are compared between the genes. Remarkably, 10 out of 12 splice sites are identical between PheOH and TyrOH and 9 out of 10 splice sites of TrpOH are conserved with TyrOH and PheOH (Figure 3). The most notable differences between the genomic organization of these hydroxylases are that the splice site between exons 1 and 2 (Glu-20 in PheOH; Thr-30 in TyrOH and non-coding sequence in TrpOH) has not been conserved and that rat TyrOH encodes 48 additional amino acids at the N-terminus compared with human PheOH [85]. These data support the notion that the hydroxylase genes constitute a gene family with a common evolutionary origin and that the evolutionary divergence is probably targeted towards the N-terminal regulatory domain.

Tertiary and quaternary structure

There is essentially no information available on the three-dimensional structure of any of the aromatic amino acid hydroxylases, and this is largely due to difficulties in the crystallization of these proteins. Crystals of PheOH have been reported but as yet no structure has been produced [86].

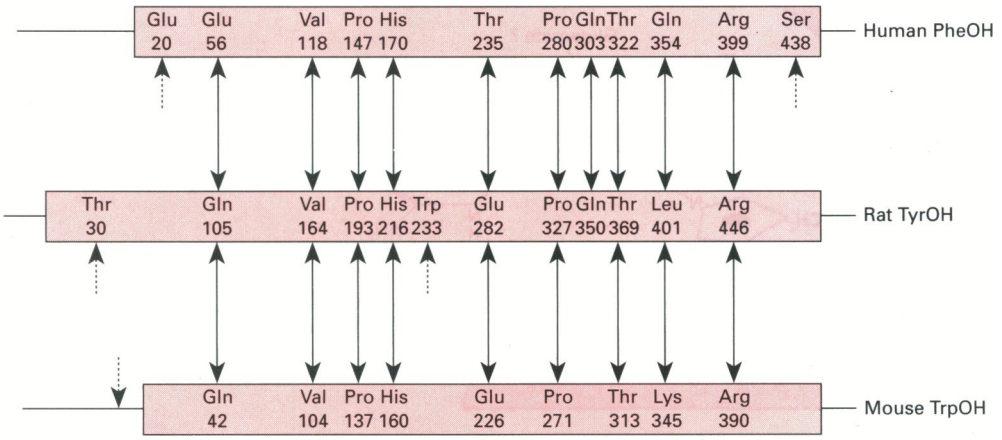

Figure 3 Conservation of intron/exon boundaries between the aromatic amino acid hydroxylases

The cDNA sequences of human PheOH, rat TyrOH and mouse TrpOH are aligned. The codon numbers and the amino acid residues at the sites of insertion of introns along the coding sequence are shown (numbering includes the initiator methionine). Conserved splice sites are shown by double-headed arrows; non-conserved splice sites are indicated by broken arrows. (Adapted from [46,85,216].)

TyrOH and TrpOH are thought to exist primarily, if not exclusively, as tetramers of identical subunits [36,87–89]. PheOH has been suggested to exist as an equilibrium of dimers and tetramers [90–92]; however, there have also been reports that it exists solely as tetramers [19,20]. Analysis of the primary sequences of the aromatic amino acid hydroxylases has shown the existence of three highly conserved regions possessing either leucine zipper or coiled coil characteristics [93]. A recent report by Lohse and Fitzpatrick [94] has suggested that the C-terminal 20 amino acids of TyrOH, which have coiled coil characteristics, may form the intersubunit binding region. However, the exact nature of the subunit–subunit interaction remains to be defined, as does the physiological significance of multimerization in each specific aromatic amino acid hydroxylase.

There is no real evidence for the existence of isoenzymes of PheOH except that which is related to post-translational modifications, i.e. phosphorylation [95]. However, there has been a report of two allelic forms of rat PheOH which differ by a threonine-to-isoleucine substitution at position 372 [96], but no differences in function or stability between these two forms could be detected. In contrast to PheOH, human TyrOH exists in at least four different isoenzyme forms (HTH1–HTH4). These are generated by alternative splicing events which appear to be restricted to the regulatory domain [48]. The distribution of these isoforms has been reported to be tissue-specific, HTH1 being predominant in the adrenal medulla, locus correuleus and substantia nigra of the brain [48,97–99].

TrpOH from different organs has different biochemical properties, which suggests that there may be several forms of this enzyme representing distinct genetic loci. These differences include divergent molecular masses, substrate specificities, turnover rates and discrepancies in isoelectric points [37,100–102]. The basis of these biochemical differences remains unclear. Levels of TrpOH mRNA are much greater in the rat pineal gland than in the brainstem despite higher activity in the latter; this suggests that different forms of TrpOH may exist in the two tissues [103]. However, cDNA has been characterized from both tissues and found to be identical [102]. These data support the hypothesis that tissue-specific differences in the properties and levels of TrpOH activity result from different post-translational events, the exact mechanism of which remains unclear but may be connected to phosphorylation.

Cofactor binding sites

All three enzymes require the cofactor BH_4 for activity, and the location of the binding site is of particular interest. Gibbs and Benkovic [104] have used a photoaffinity-labelled pterin analogue to probe the pterin binding site of PheOH. This azidopterin derivative {2,6-diamino-5-[(3-azido-6-nitrobenzylidine)amino]-pyrimidin-4-one; ANBADP} was shown to be a competitive inhibitor of PheOH and bound irreversibly to the enzyme. Three tryptic peptides of the treated enzyme were shown to contain a common seven-residue sequence (residues 193–199 in the rat PheOH sequence). The stoichiometry of cofactor binding was approx. 1 molecule per enzyme subunit, with most of the label being associated with Lys-199 and a lesser quantity with Lys-195. It was also noted that immediately adjacent to this proposed pterin-binding region was a His-Xaa-Xaa-Xaa-Glu-Xaa-Xaa-His motif. This triad of two imidazoles and one carboxylate is reported to be involved in the binding of non-haem iron [105]. Recent studies on TyrOH have converted Lys-241 (this is equivalent to Lys-195 in PheOH) to an alanine and have shown no kinetic differences between the mutant and wild-type enzymes [106]. This does seem to indicate that the photoaffinity approach using a pterin analogue may have given some misleading data; it may, however, reflect subtle differences between these enzymes. Although ANBADP satisfied the criteria for a specific photoaffinity activatable label, it contained bulky nitro and azido groups located at the 6′ position in BH_4 (Figure 1). The production of a pteridine adsorbent coupled to a chromatography matrix via the 5′ position has been used to purify PheOH [107,108]. The success of this purification procedure and the ability of synthetic analogues of BH_4 (substituted at the 6′ position) to remain functionally active does suggest that this region of the pterin molecule is not important in binding to the enzyme. It may be that both Lys-195 and Lys-199 were close enough in the three-dimensional structure to react with ANBADP at the pterin-binding site. Although the region bracketed by Lys-195 and Lys-199 is conserved amongst PheOHs, the degree of conservation amongst the 13 aromatic amino acid hydroxylases sequenced to date [82] is not significant, in contrast to what would be expected if these residues were defining a conserved function such as pterin binding.

An alternative approach used an anti-idiotypic antibody that mimics the pterin and binds PheOH at the pterin-binding site [109]. The authenticity of the anti-idiotypic antibody was tested by (1) its ability to generate anti-pterin antibodies when reinjected into mice; (2) confirmation that anti-idiotypic antibody binding was prevented by a competitive inhibitor of PheOH; and (3) its ability to bind to a large number of pterin-requiring enzymes. The pterin-mimicking antibody has been shown to react with PheOH, TyrOH, TrpOH, dihydropteridine reductase, dihydrofolate reductase and sepiapterin reductase, which suggests strong structural homology amongst the pterin sites of these enzymes, despite diverse functions [110]. The antibody binding site was sequentially localized to a V8-proteinase-digested fragment of PheOH (estimated to span Leu-128 to Glu-305) and then to a 27-amino-acid peptide (His-264 to His-290) which is highly conserved amongst the aromatic amino acid hydroxylases [82]. More recently, site-directed mutagenesis of this region has identified Glu-286 as a critical residue in the function of the pterin cofactor in PheOH [111]. Whether or not these two proposed pterin binding regions are in close proximity in the enzyme three-dimensional structure will probably only be known when an X-ray crystal structure of an aromatic amino acid hydroxylase becomes available.

Iron-binding site(s)

Values for the exact stoichiometry of iron binding to PheOH have been variable but it is now generally accepted that one molecule of functional iron is bound per subunit [112,113]. Physiochemical studies of TyrOH have been limited by the small amount of pure enzyme available. However, a large-scale purification of TyrOH from bovine adrenal medulla has given a value of 0.6–0.8 mol per subunit [27]. More recent studies using recombinant human TyrOH, which was purified as a metal-free apoenzyme (apo-HTH1), showed the enzyme to be rapidly activated by the incorporation of stoichiometric amounts of Fe^{2+} [114]. TrpOH does require iron for expression of catalytic activity, in that Fe^{2+} stimulates purified TrpOH primarily by increasing the reaction velocity [89,115]. However, the exact nature/stoichiometry of iron binding is not known, largely due to the instability associated with this enzyme.

Attempts to identify iron-binding sites have been mainly restricted to studies on rat PheOH, in which His-285 and His-290 are proposed to form the iron-binding site(s) [113]. By site-directed mutagenesis of these histidine residues and expression in insect cells it was demonstrated that the mutant proteins were

unable to bind iron or to hydroxylate phenylalanine. Both of these residues lie within the proposed pterin-binding region identified by Jennings et al. [109]. As stated earlier, a conserved motif of His-Xaa-Xaa-Xaa-Glu-Xaa-Xaa-His is suggested to be involved in the binding of non-haem iron through a triad arrangement of two imidazoles and one carboxylate (Figure 2). Within the proposed pterin-binding site (His-264 to His 290) there are four histidines and two glutamate residues, but as yet there is no evidence for His-264, His-271, Glu-280 or Glu-286 being involved in iron binding. It is believed that iron bound to PheOH exists in distinct environments [116] but what proportion of the iron is involved in catalysis is open to speculation. A recent report by Shiman et al. [67] has used specific iron chelators to probe the enzyme's functional iron and shown it to be in a hydrophobic pocket and solvent accessible.

Phenylalanine has been shown to interact with the iron at the active site of PheOH. Using EPR and NMR, both phenylalanine and noradrenaline were reported to bind closely to the paramagnetic iron, and catecholamines (which are potent inhibitors of both PheOH and TyrOH) were able to displace phenylalanine from the active site [117,118]. More recent studies have measured changes in the accessibility of water to the enzyme's iron upon binding of phenylalanine, noradrenaline and lysolecithin. The results obtained showed that a water molecule co-ordinated to the Fe^{III} is displaced upon binding of phenylalanine or noradrenaline, but not lysolecithin, which suggests that lysolecithin mediates its activation of PheOH at another site [119]. The Fe^{III} at the active site is reported to be in direct co-ordination with catecholamines [117] and there is evidence to suggest that a residue of pK_a 5.1 (cf. glutamate/aspartate residues) is directly involved in binding. A similar result concerning the interaction of noradrenaline with TyrOH has been observed [120,121]. This may represent a common structural motif at the metal centre of aromatic amino acid hydroxylases and may be related to recent studies which have identified Glu-286 as being of critical importance in the binding of the pterin cofactor [111].

It is worthy of note that a homologous PheOH enzyme from *Chromobacterium violaceum* has been reported to contain copper instead of iron with a stoichiometry of 1 mol of copper/mol of enzyme subunit at its catalytic site [122]. More recent experimentation by the same group has concluded that copper, and indeed any active metal species, is not required for catalysis [123]. The implications that this metal-independent pterin-mediated hydroxylation reaction has for the mechanism of hydroxylation in the equivalent mammalian enzymes are not known.

Substrate binding sites

Little is known about the amino acids responsible for substrate binding in any of the aromatic amino acid hydroxylases. Most effort has been directed towards PheOH, but studies have been complicated by the fact that substrate analogues, including non-aromatic amino acids, can activate the hydroxylases and serve as substrates for the activated enzyme [124]. The exact stoichiometry of phenylalanine binding to PheOH has been difficult to measure. In the native enzyme it has been reported that 1.5 mol of phenylalanine binds per enzyme subunit [125]. This provided the first physical evidence for the existence of two phenylalanine-binding sites, with a regulatory or allosteric site which is distinct from the catalytic site [126–129]. Enzyme activated by *N*-ethylmaleimide was able to bind 1 mol of phenylalanine/mol of enzyme subunit, which seems to indicate that the site that binds 0.5 mol/mol of subunit is the regulatory site [125]. It could be expected that the phenylalanine site involved in catalysis is at the active site, as defined by pterin-binding and iron-binding studies [104,109,113], and the regulatory site is in the N-terminal domain as defined by homology, proteolysis and phosphorylation data (see 'Regulation' section below). As yet no residues involved in substrate binding in any aromatic amino acid hydroxylase have been clearly defined. However, it has been reported that a PKU mutation deleting isoleucine at residue 94 results in a defective enzyme with a reduced affinity for phenylalanine [130]. The consideration of other enzymes that metabolize phenylalanine, tyrosine or tryptophan may provide an alternative route to defining substrate specificity in the aromatic amino acid hydroxylases. A recent report has defined a hexapeptide sequence motif (i.e. Phe-Val-His-Ala-Ala-Arg) involved in substrate recognition in phenylalanine dehydrogenase [131].

REGULATION

It is clear that the physiological regulation of PheOH is different to that of TyrOH or TrpOH. As phenylalanine is both an essential amino acid and is toxic via its metabolites, the tight regulation of PheOH activity is a physiological necessity to avoid wasteful consumption of the phenylalanine pool and to protect the developing brain from possible damage. In contrast to PheOH, it is apparent that neither TyrOH nor TrpOH is involved in the catabolism of their respective substrates, but catalyse hydroxylation reactions in neurotransmitter synthesis. As such, dietary intake of these substrates may be expected not to play a major regulatory role, but rather serves the requirements of the organism's nervous system.

Methods of *in vitro* activation

The enzyme PheOH can be activated by a variety of *in vitro* modifications, the physiological significance of which is not always clear. These include interaction with phospholipids (e.g. lysolecithin) [132], alkylation of a single thiol group [125] and partial proteolysis [91,132]. Lysolecithin is reported to induce a conformational change, and it seems to effect the same functional changes in the enzyme as does phenylalanine [133] but does not appear to compete for the same activation site (see 'Substrate-induced activation' below). Chymotrypsin reduces the size of PheOH from 52 kDa to 36 kDa by the removal of the N-terminal 11 kDa fragment, which includes the phosphorylation site, and the C-terminal 5 kDa fragment [91]. The 36 kDa domain that remains is 30-fold more active than native PheOH when assayed in the presence of BH_4, suggesting that the regulatory domain that mediates PheOH inhibition is located in the N-terminal third and/or the extreme C-terminus of the enzyme. The so-called catalytic core of PheOH has been further characterized and the minimum catalytic fragment is from Leu-142 to Phe-410 [111].

Treatment of rat PheOH with *N*-ethylmaleimide modifies a single thiol group, and the resultant enzyme appears to have the same characteristics as enzyme activated by partial proteolysis or lysolecithin [125]. The residue involved in this activation has been identified by labelling the reactive thiol with fluorescein [104]; 1 mol of fluorescein was bound per PheOH subunit and resulted in activation to at least 82% of the level caused by phenylalanine. The label was localized to Cys-237, which is conserved in rat, human, mouse and *Drosophila melanogaster* PheOHs (not bacterial PheOH) but is replaced by an arginine in all TyrOHs and TrpOHs studied to date. This might reflect differences in the allosteric regulation of the aromatic amino acid hydroxylases, and it could be that Cys-237 is at or near to the regulatory phenylalanine-binding site in the three-dimensional structure of PheOH. PheOH has been shown to be activated by alkaline pH. Results obtained are consistent with there being an

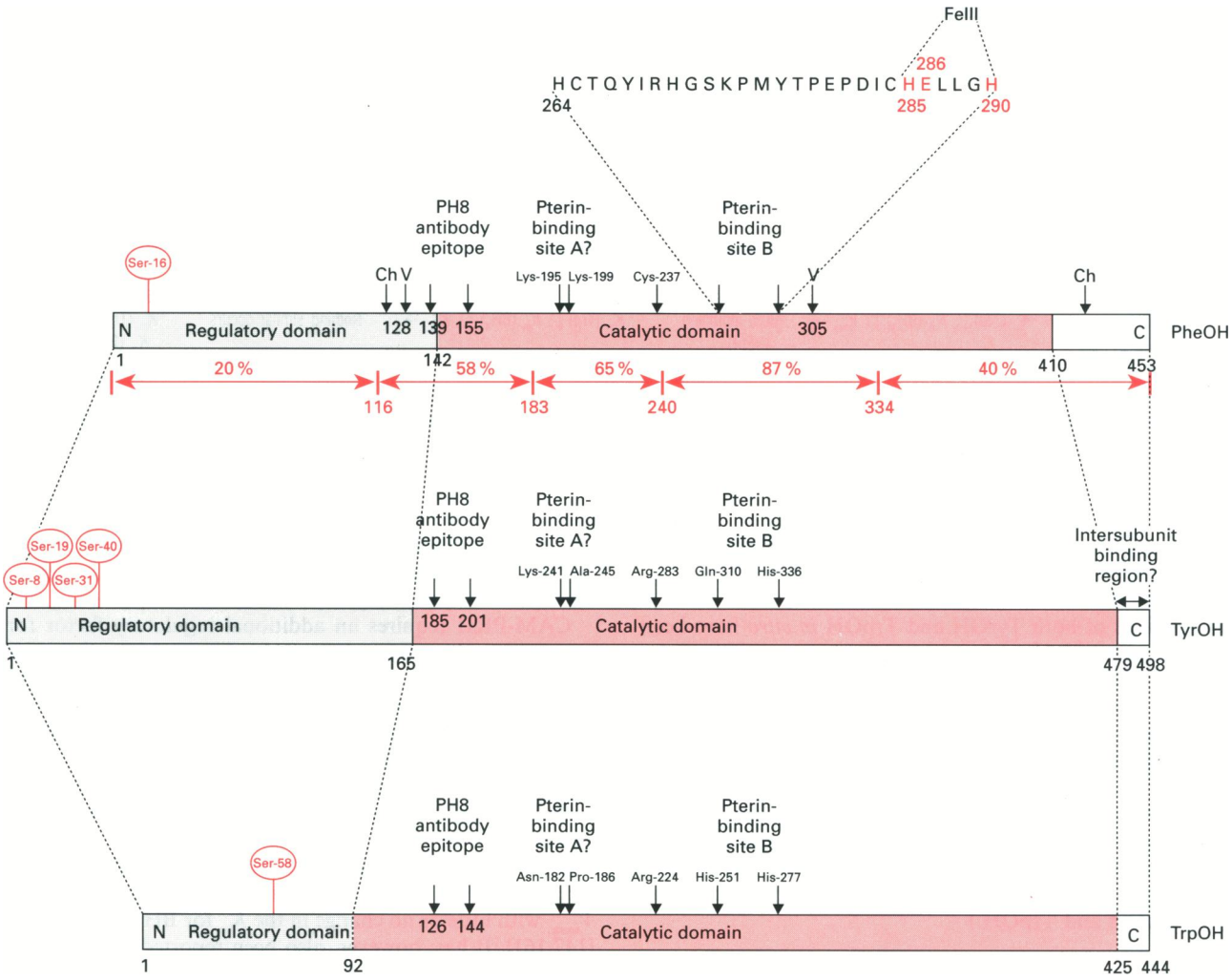

Figure 4 Important structure/function correlations between the three aromatic amino acid hydroxylases

Data were obtained primarily with rat PheOH, rat TyrOH and human TrpOH. The regulatory domain, the catalytic domain (pink region), as defined by deletion mutagenesis, and the proposed inter-subunit binding domain proposed in TyrOH are shown. The catalytic domain for human TrpOH is delineated by broken lines, relating to it having very low activity [144]. Two reported pterin-binding sites are indicated, i.e. site A [104] and site B [109], as are the approximate positions of the cleavage sites for chymotrypsin (Ch) and V8 protease (V), the PH8 epitope, the reactive thiol group (Cys-237 in PheOH), and a proposed iron-binding site (His-285, His-290 and possibly Glu-286 in PheOH). The serine residues in the regulatory domain proposed to be involved in regulation by phosphorylation are also indicated. Values for percentage sequence identity between PheOH and TyrOH calculated for segments of the protein are shown (red) to highlight correlations between conserved structural features and sequence identity [223]. All numbering of sequences includes the initiator methionine.

ionizable group with a pK_a of approx. 8.0 being involved in the substrate-induced activation of the enzyme [133,134].

The initial findings relating to the activation of PheOH were extended to include TyrOH and TrpOH, and it was shown that they also can be activated by partial proteolysis and phospholipid [135–140], but they appear to be inactivated by thiol modification [115,141]. In TyrOH a regulatory fragment from residues 1 to 158 is phosphorylated at the same site as the holoenzyme but has no catalytic activity, whereas the catalytic fragment from residues 155 to 498 is fully active [142]. Further delineation of the domain structure of TyrOH has shown the catalytic core to be between residues 165 and 479 [143]. The catalytic domain of mouse TrpOH has similarly been shown to be encoded by the C-terminal 380 amino acids [54]. Further definition of the domain structure of TrpOH has recently been completed and suggests that the catalytic domain may be between residues 92 and 425 [144] (Figure 4). However, the activity obtained with this and all other deletion mutants was very low or non-detectable, which suggests that the functioning of the N- and C-termini of TrpOH may be different from that of TyrOH and PheOH.

The changes in kinetic properties induced by these treatments of PheOH are significantly different from those induced in TyrOH and TrpOH. Treatment of PheOH with lysolecithin or proteolysis results in a 20–40-fold increase in activity only when assayed in the presence of BH_4. The activation is predominantly due to an increase in $V_{max.}$, with the K_m for phenylalanine being reduced by 50% and the K_m for BH_4 being largely unaffected [91,125,132]. There is also a dramatic broadening of substrate specificity, an increase in sensitivity to phenylalanine inhibition at concentrations greater than 0.2 mM, and the relationship between initial velocity and phenylalanine concentration is changed from a sigmoidal to a hyperbolic relationship [124,132]. The activity of both TyrOH and TrpOH is only increased 2–4-fold by treatment with phospholipid or partial

Table 2 Effects of various treatments on the kinetic constants of the aromatic amino acid hydroxylases

↑, ↓ and → indicate an increase, decrease or no change respectively in the kinetic constants. Abbreviation: NEM, *N*-ethylmaleimide. See the text for further details and for references.

	Phospholipid	Proteolysis	Thiol modification	Anions/polyanions	Preincubation with substrate	Phosphorylation
PheOH	K_m (Phe) ↓ K_m (BH_4) →; $V_{max.}$ ↑	As for phospholipid	As for phospholipid	No reported effects	50% activation with 0.06 mM Phe	K_m (Phe) →; K_m (BH_4) →; $V_{max.}$ ↑ or →
TyrOH	K_m (Tyr) →; K_m (BH_4) ↓; $V_{max.}$ →	K_m (Tyr) ↓; K_m (BH_4) ↓; $V_{max.}$?*	100% inhibition (NEM)	K_m (Tyr) →; K_m (BH_4) ↓; $V_{max.}$ ↑	Nothing similar occurs	pH 6.0: K_m (BH_4) ↓; $V_{max.}$ →; pH 7.0: K_m (BH_4) ↓; $V_{max.}$ ↑
TrpOH	K_m (Trp) →; K_m (BH_4) ↓; $V_{max.}$ →	K_m (Trp) ↓; K_m (BH_4) ↓; $V_{max.}$ →	60% inhibition (NEM)	K_m (Trp) ↓; K_m (BH_4) ↓; $V_{max.}$ →	Nothing similar occurs	K_m (Trp) ↓ or →; K_m (BH_4) ↓; $V_{max.}$ ↑ or →

* No conclusion.

proteolysis and is manifested predominantly in a decrease in the K_m for the pterin cofactor rather than an increase in $V_{max.}$ [135,137,139,140]

The activities of both TyrOH and TrpOH *in vitro* have been shown to be affected by both anions and polyanions (e.g. heparin, RNA, DNA) and this results in a decrease in the K_m for BH_4 [26,139,145]. The activation of purified TyrOH by RNA or DNA has been investigated using the apolar fluorescent probe 8-anilino-1-naphthalenesulphonic acid and shown to coincide with conformational changes within the enzyme [146]. Study of these *in vitro* activation procedures has provided an insight into the allosteric regulation of these enzymes; however, it remains to be shown if they have any physiological significance. (See Table 2 for a comparison of the kinetic effects of the above factors on PheOH, TyrOH and TrpOH.)

Modification by phosphorylation

There is sufficient evidence to show that all three aromatic amino acid hydroxylases can be phosphorylated and that this is a physiologically relevant post-translational modification. The phosphorylation of purified rat PheOH has been shown to be dependent on cyclic AMP-dependent protein kinase (PKA) [147] or Ca^{2+}/calmodulin-dependent protein kinase II (CAM-PKII) [148]. Phosphorylation *in vivo* was shown by the isolation of phosphorylated forms of PheOH from rats treated intravenously with glucagon, which is known to increase the intracellular levels of cyclic AMP, giving direct evidence that phosphorylation occurs in the liver of the whole animal [149]. A monoclonal antibody specific for the phosphorylated form of PheOH [150] has been used to investigate hormonal effects on the phosphorylation of PheOH in the rat kidney. The most potent effector of enzyme phosphorylation was found to be parathyroid hormone, which is known to stimulate the production of cyclic AMP in rat proximal kidney tubules [151,152].

The phosphorylation of TyrOH appears to be associated with several factors; these include environmental signals (e.g. cold), nerve stimulation and several first messengers (e.g. cholinergic and peptinergic agonists). Detailed study has also shown the tight coupling of phosphorylation of TyrOH, to different extents, with a series of cellular second messengers including cyclic AMP, diacylglycerol, Ca^{2+} and three types of protein kinase (PKA, Ca^{2+}/phospholipid-dependent kinase and CAM-PKII) [4,153]. TrpOH activity is increased in a Ca^{2+}/calmodulin-dependent manner [154] and CAM-PKII has been shown to phosphorylate the enzyme [155]. There is evidence to suggest that cyclic nucleotides increase TrpOH activity [156,157]; however, a role for PKA has not clearly been established.

CAM-PKII requires an additional regulatory factor for the activation of TyrOH and TrpOH [158]. Multiple distinct forms of a brain-specific activator protein (i.e. 14-3-3 protein) have been identified and shown to share a common structural feature in containing an extremely acidic C-terminal domain [159,160]. It is believed that this C-terminal domain interacts with the phosphorylated forms of TyrOH and TrpOH and mediates the stimulus-coupled long-term regulation of monoamine biosynthesis.

The effect of phosphorylation on PheOH kinetics is somewhat controversial. It has been reported to result in an increase in the $V_{max.}$ with little or no change in the K_m for BH_4 or phenylalanine [147,161]. It has, however, also been reported that phosphorylation does not affect the $V_{max.}$ but merely allows PheOH to be more easily activated by phenylalanine [162,163] (see section on Substrate-induced activation, below). In contrast to PheOH, the cyclic AMP-dependent phosphorylation of either rat or bovine striatal TyrOH varies with pH. At pH 6.0 there is little change in $V_{max.}$ and a large decrease in the K_m for the pterin cofactor, whereas at pH 7.0 there is a large increase in $V_{max.}$ and only a small decrease in the K_m for the pterin cofactor [164]. The Ca^{2+}-dependent phosphorylation of TrpOH has been reported to decrease the K_m for the pterin cofactor with little change in the K_m for tryptophan, but [140,165,166] there is no agreement as to whether this type of activation also increases the $V_{max.}$. There is, however, general agreement that phosphorylation of both TyrOH and TrpOH results in a decrease in the K_m for the pterin cofactor (Table 2).

The phosphorylation site of PheOH has been identified as Ser-16 [167] (Table 3) and was subsequently shown to be the unique and common residue phosphorylated by either PKA or CAM-PKII [148,168]. The sequence surrounding the phosphorylatable residue is typical of many substrates of PKA in that it is preceded by an arginine, two residues N-terminal to Ser-16 [167,169,170]. Corresponding model phosphopeptides, spanning Ser-12 to Gly-19, had significantly higher K_m values for the protein kinase than whole PheOH. This suggests that the local structure surrounding Ser-16 may be of importance in recognition by the protein kinase. It is worthy of note that Ser-16 has been reported to lie within a predicted β-turn secondary structure [43]. A monoclonal antibody specific for the phosphorylated form of human PheOH has been shown to recognize a linear epitope spanning Leu-15 to

Table 3 Phosphorylation of the aromatic amino acid hydroxylases

See the text for further details and for references.

	Phosphorylation site(s)	Protein kinase	Activator 14-3-3 protein
PheOH	Ser-16	PKA	Nd
	Ser-16	CAM-PKII	Nd
TyrOH	Ser-8	Not known	Nd
	Ser-19	CAM-PKII	Yes
	Ser-31	ERK1 and ERK2	Nd
	Ser-40	PKA	Nd
TrpOH	Ser-58*	CAM-PKII	Yes
	Ser-58*	PKA	Nd

* A direct link between phosphorylation, a protein kinase and changes in TrpOH activity remains to be clearly established. Abbreviation: Nd, not determined.

Gly-19 [150], including the phosphate-acceptor residue Ser-16. Recent studies have subjected Ser-16 in rat PheOH to site-directed mutagenesis. Replacement by a negatively charged glutamate residue had the same effect on the enzyme as phosphorylation, whereas replacement with an uncharged alanine residue resulted in an enzyme essentially the same as the unphosphorylated form. These results suggested that modification by phosphorylation involve electrostatic interactions [171].

TyrOH is reported to have four phosphorylation sites (Ser-8, -19, -31 and -40) in intact rat tissue; however, physiological stimuli only induce phosphorylation of Ser-19, Ser-31 and Ser-40 [172–175]. *In vitro*, Ser-19 is a substrate for CAM-PKII, Ser-40 is a substrate predominantly for PKA [176–179], and Ser-31 is phosphorylated by two microtubule-associated protein kinases, extracellular signal-related protein kinase ERK1 and ERK2 [180]. These two extracellularly regulated protein kinases are involved in a pathway in which diacylglycerol and protein kinase C function upstream of the ERK1- and ERK2-dependent phosphorylation of Ser-31. Activation by cyclic AMP-dependent phosphorylation of Ser-40 is the only well documented effect of phosphorylation on TyrOH activity. This phosphorylation site appears to mediate an inhibitory effect on TyrOH activity [181] and its phosphorylation is believed to be of regulatory importance in relieving the inhibitory effects of catecholamines on TyrOH [182]. The relevance of the other phosphorylation sites has yet to be determined. All four phosphorylation sites are in the N-terminal domain, which does not show any significant sequence similarity to PheOH or TrpOH [172].

Molecular cloning experiments have suggested that Ser-58 (which is within the proposed N-terminal domain) [54,144] of TrpOH constitutes a good candidate for cyclic AMP-dependent phosphorylation [51–54]. Outside the N-terminal domain there are two other potential phosphorylation sites at Ser-260 and Ser-443 which are conserved between rabbit, rat, mouse and human TrpOH. These are potential sites for Ca^{2+}/calmodulin-dependent phosphorylation [183], which is in accordance with *in vivo* and *in vitro* studies by Kuhn et al. [154] and Nagatsu et al. [184]. A direct link between phosphorylation of TrpOH and any protein kinase remains to be established (Table 3).

A protein phosphatase isolated from rat liver was found to catalyse the dephosphorylation of PheOH [185] and has been classified as a type 2A phosphatase. Since the enzyme is mainly cytosolic and is the major phosphatase in liver extract with activity towards phosphorylated PheOH, it may be the major phosphatase acting *in vivo* [186,187]. Similar results have been obtained for bovine TyrOH in that an apparently homogeneous sample was contaminated with significant amounts of phosphatase type 2A, which is the major phosphatase present in bovine adrenal medulla [188].

Substrate-induced activation

Phenylalanine plays a major role in the regulation of PheOH, and it is reported to activate the enzyme [133]. This substrate-mediated regulation can occur by three distinct but related mechanisms. Phenylalanine can activate PheOH directly by inducing a conformational change; this has been shown by UV spectroscopy [189] and by an increase in the surface hydrophobicity of PheOH in the presence of substrate [20]. The substrate has also been reported to enhance the cyclic AMP-dependent phosphorylation of PheOH, which in turn sensitizes the enzyme to further allosteric activation by phenylalanine [162,163,168]. Finally, in some species phenylalanine can elicit the release of glucagon from the pancreas, leading to increased cyclic AMP levels and thus enhancing phosphorylation [149]. The activation of PheOH by phenylalanine has been shown *in vivo* [162,190] but its connection with glucagon-mediated phosphorylation is unclear. Tipper and Kaufman [190] have noted a large stimulation of hydroxylase activity and incorporation of radioactive phosphate into PheOH on glucagon treatment, which was significantly greater than that induced by treatment of rats with phenylalanine alone. There is no evidence as yet to suggest that TyrOH and TrpOH are regulated by substrate-activation mechanisms similar to those that regulate PheOH.

It is believed that PheOH exists as an equilibrium of high-activity and low-activity conformations [163,191] upon which phenylalanine and BH_4 have opposing effects with regard to the phosphorylation state of the enzyme. A more recent model by Xia et al. [192] proposes that the *in vivo* regulation of PheOH is dependent primarily on the actions of phenylalanine and BH_4, with phosphorylation being of less physiological importance. Phenylalanine is proposed to act as a positive effector and BH_4 as a negative effector that blocks phenylalanine activation by forming an inactive BH_4–enzyme complex. The formation of this complex controls the metabolic availability of BH_4 so that the free concentration is much less than the total amount present. The sequestering of BH_4 by PheOH and its release mediated by phenylalanine is reported as a novel control mechanism [192,193]. The relationship *in vivo* between phenylalanine-mediated activation, inhibition by BH_4, cyclic AMP-dependent phosphorylation and the action of glucagon is summarized in Figure 5.

Native PheOH has been suggested to exist as a mixture of dimers and tetramers, the equilibrium of which can be shifted to the tetrameric form by incubation with phenylalanine [90–92]. This phenylalanine-induced change in the oligomeric structure of PheOH is of interest in relation to enzyme activation. The literature is, however, controversial with respect to the catalytic activities of the dimeric and tetrameric forms of PheOH. Parniak [92] has reported that both oligomeric forms are active, although the specific activity of the tetramer is five times greater than that of the dimer. Shiman et al. [129], on the other hand, report that only the tetrameric form is active. A model has been proposed by Parniak that provides an explanation for the reported stoichiometry of 1.5 mol of phenylalanine/subunit and also the observation that the dimeric and not the tetrameric form of PheOH shows non-cooperative binding of phenylalanine [125].

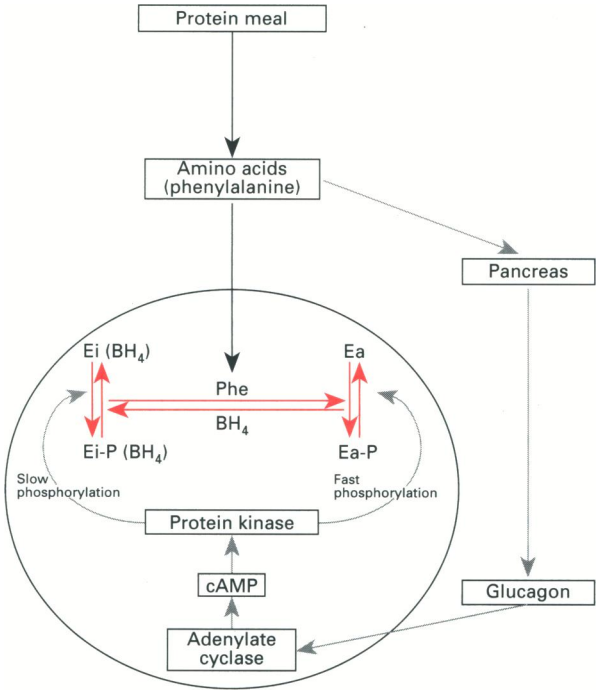

Figure 5 ***In vivo*** **regulation of PheOH**

A scheme showing the effects of glucagon, BH_4 and phenylalanine on the interconversion of the various forms of rat liver PheOH *in vivo*. Ei represents an inactive or a low-activity conformation of the hydroxylase whereas Ea is a high-activity conformation. Ei-P and Ea-P are the phosphorylated forms of these two conformations. The pathway leading to the phosphorylation of PheOH is shown by grey arrows, whereas direct activation by phenylalanine is shown by a black arrow. The scheme assumes that Ea is a better substrate for phosphorylation than Ei and that phosphorylation of Ea is much faster than that of Ei. BH_4 is shown bound to an inactive or low-activity enzyme. The release of free BH_4 and activation of the enzyme is mediated by phenylalanine binding to the proposed allosteric site. cAMP, cyclic AMP. (Adapted from [78,192].)

The model predicts that the allosteric phenylalanine-binding sites are absent or have low affinity in the dimer, but upon formation of tetramers the sites are formed or become functional [194]. It must be remembered that the existence of different oligomeric forms of PheOH could be explained by differences in the state of denaturation of the enzyme, and therefore such a model must be treated with a certain degree of caution as it may not represent the situation *in vivo*. Shiman has proposed an alternative model in which phenylalanine binds to one activation site per enzyme subunit. This site is physically distinct from the catalytic site and its binding of phenylalanine is co-operative. Phenylalanine remains at this site during catalysis and is not hydroxylated; its removal in turn causes loss of enzyme activity [129].

A monoclonal antibody, PH1, has been reported to recognize an epitope associated with phenylalanine activation of PheOH. The inhibition of PheOH by PH1 is non-competitive with respect to the pterin cofactor and is accompanied by the abolition of the phenylalanine-associated co-operativity of PheOH. This suggests that PH1 recognizes an epitope associated with phenylalanine binding in the regulatory domain of PheOH and prevents the transfer of conformational information concerning this substrate binding to the catalytic domain [80,195].

It has been postulated that the activation of the aromatic amino acid hydroxylases involves interactions between the catalytic domain and the regulatory domain which mediate access to the enzyme active site [65,91] (see above). There is significant indirect evidence for an inhibitory effect of the regulatory domain on the catalytic domain; however, there is as yet no direct evidence for such interactions. The removal of the regulatory domain [91,135,139] and the alkylation of a thiol group (Cys-237 in PheOH) [125] are both postulated to expose the active site in the catalytic domain. Phospholipids [132,137,140] and polyanions [26,139,145] are imagined to either remove or neutralize the interactions between the two domains. As the catalytic domain is predominantly hydrophobic and the regulatory domain is hydrophilic [43], it could be proposed that amphipathic phospholipid molecules intercalate between the two domains. The regulatory mechanisms that are believed to operate *in vivo* (i.e. phosphorylation [4,147,148,153,155–157] and substrate activation in the case of PheOH [133]) could be expected to induce a conformational change so as to expose the enzyme active site. It should be remembered that the three aromatic amino acid hydroxylases do show subtle differences in their response to the above modifications (Table 2) and these probably reflect differences in their allosteric regulation. Figure 6 attempts to summarize some of the information primarily concerning the activation of PheOH and its proposed effect on domain interactions within the hydroxylases.

Inhibition

All three aromatic amino acid hydroxylases are inhibited by catecholamines [2]. In the case of PheOH and TyrOH this has been shown to be due to the direct co-ordination of catechols (i.e. dopamine, noradrenaline and adrenaline) to the Fe^{3+} at the enzyme active site [118,196]. Although the role of these molecules as feedback inhibitors and regulators of TyrOH is considered to be of physiological significance [196,197], it is not thought that they have a similar role in the regulation of PheOH *in vivo*. It has been reported that the extracellular concentration of catecholamines required to give 50% inhibition of PheOH activity in hepatoma cells was much greater than the concentration required to affect hepatocytes by binding to cell surface receptors [198,199].

The inhibition of human TyrOH by catecholamines 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 [121]. Phosphorylation of Ser-40 in TyrOH is reported to cause a dramatic decrease in the binding affinity for catecholamines [121] and an increase in the affinity for the cofactor [200]. Furthermore it has been shown that removal of the N-terminal 30 amino acids from bovine TyrOH causes an increase in the K_i for dopamine [201]. Together, these data suggest that there is a mutual interaction between the catalytic and regulatory domains of TyrOH. The activation of the enzyme by phosphorylation of Ser-40 and the inactivation by binding of catecholamines are related phenomena and probably represent important *in vivo* regulatory mechanisms.

In contrast to PheOH, the hydroxylation of tyrosine by TyrOH shows pronounced inhibition at high substrate concentrations; this has been reported in TyrOH isolated from adrenal gland [202], brain tissue [203] and pheochromocytoma cells [204] and in the enzyme expressed in heterologous systems [200,205]. This inhibition is evident at physiological concentrations of tyrosine and is likely to be of importance in the regulation of catecholamine synthesis *in vivo*.

Besides the role of the cofactor in the catalytic reaction, changes in its physiological concentration have significance in the regulation of PheOH specifically. BH_4 has been reported to inhibit the activation of PheOH by a reversal of the phenylalanine

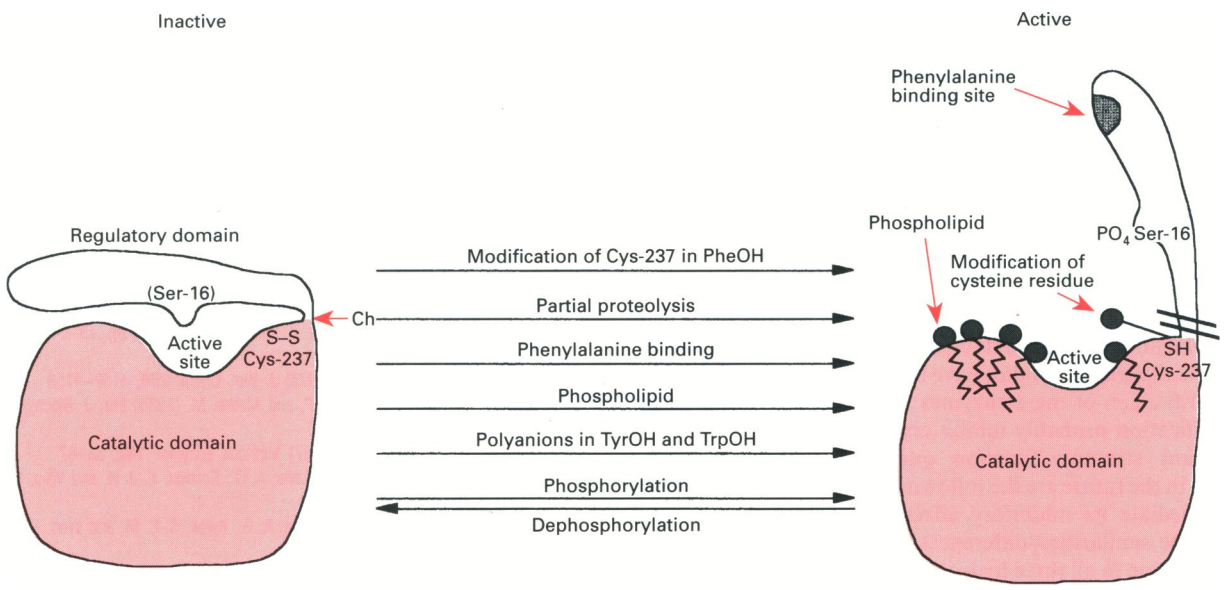

Figure 6 Model of the allosteric regulation of PheOH

A proposed scheme for the allosteric regulation of aromatic amino acid hydroxylases is shown, using data predominantly obtained from the study of PheOH. The enzyme may be activated by (i) thiol modification of Cys-237 in PheOH [125], (ii) proteolytic removal of the regulatory domain by chymotrypsin (Ch) [91,135,139], (iii) preincubation of PheOH with phenylalanine [133], (iv) treatment with phospholipid [132,137,140], (v) treatment of TyrOH and TrpOH with anions or polyanions [26,139,145] and (vi) modification by phosphorylation and dephosphorylation [4,147,148,153,155–157]. The regulatory domain is represented stylistically as a mobile structure able to mediate access to the active site of the catalytic domain. It is suggested that all the above-mentioned modifications serve to affect the interaction between these two domains. The phosphorylation site (i.e. Ser-16) and the postulated allosteric phenylalanine binding site of PheOH are indicated.

activation process [127,133]. The proposed conformational change induced by phenylalanine, as measured by spectrofluorimetry, is reported to be reversed by the binding of BH_4 to PheOH [189,206–208]. In addition, BH_4 has been shown to inhibit activation by lysolecithin [133] and proteolytic activation [91,209] and, in contrast to phenylalanine, BH_4 has been reported to be a negative effector of the rate of cyclic AMP-dependent phosphorylation of PheOH *in vitro* [163,191]. The importance of BH_4 as a negative effector of PheOH has recently been reassessed and a model has been proposed where free BH_4 and the formation of an enzyme–BH_4 complex have specific effects on enzyme activity and regulation [192,193] (see 'Substrate-induced activation' section above).

The inhibitory effects of BH_4 also exert their influence on another recently identified enzyme, GTP cyclohydrolase 1 [210] (Figure 1). This enzyme is the rate-limiting enzyme in the synthesis of BH_4 and is subject to feedback inhibition. This inhibition is dependent on BH_4 and another protein, P35. The inhibition is specifically reversed by phenylalanine and provides an explanation for the high BH_4 levels observed in hyperphenylalaninaemia patients, as they are unable to mediate the inhibition by phenylalanine of BH_4 production [211,212].

Transcriptional regulation

Little is known about the transcriptional regulation of the aromatic amino acid hydroxylases. The recent isolation of the promoter sequences of human PheOH has revealed the presence of several putative regulatory sequences.The promoter region was reported to lack a TATA box but to contain a variety of sequences required for interaction/regulation by multiple transcription factors [213]. The reported presence of partial glucocorticoid response elements, partial cyclic AMP response elements and activator protein 2 sites is in agreement with some of the regulatory response mechanisms which operate in PheOH gene expression. The level of PheOH is controlled by glucocorticoids, which appear to exert their regulatory effect on PheOH expression at the transcriptional level [13,214].

A role for cyclic AMP and glucocorticoids in the expression of TyrOH has been reported. By fusing the 5′ flanking region of TyrOH to chloramphenicol acetyltransferase it was demonstrated that both glucocorticoid and cyclic AMP stimulate transcriptional activity of this gene. Furthermore the sequences required for a response to cyclic AMP are within 272 bp of the translation start site [215]. Actual transcriptional regulation of TrpOH has not been demonstrated directly, primarily because mRNA is present in such low levels in the brain [51–54]. The promotor region of mouse TrpOH has been characterized but no glucocorticoid or cyclic AMP response elements have as yet been identified [216].

Phenylalanine-stimulating protein (also known as pterin 4a-carbinolamine dehydratase) was first identified by Kaufman [217] (Figure 1) and has subsequently been shown to be active in the recycling of the cofactor through the conversion of 4a-hydroxy-BH_4 into quinonoid dihydrobiopterin [218]. A defect in this enzyme has been shown to result in mild hyperphenylalaninaemia with defective cofactor metabolism and has been postulated to be responsible for the production of 7-biopterins found in certain mild cases of hyperphenylalaninaemia [212]. This protein has been characterized from rat and human and found to be identical [219,220] to human dimerization cofactor, which is required for the correct functioning of hepatocyte nuclear transcription factor-1α [221]. It may be that 4a-carbinolamine dehydratase is involved not only in the recycling of BH_4 but also in the regulation of PheOH gene expression in a similar way to human dimerization cofactor. Studies on the

structure and function of the PheOH promoter have identified at least two protein binding sites essential for the regulation of transcriptional activity [222]. However, as yet no tissue-specific transcription factor binding sites have been identified. It is possible that this is because PheOH gene expression employs non-DNA binding factors to confer tissue specificity, and an obvious candidate for such a co-activator is pterin 4a-carbinolamine dehydratase.

CONCLUSIONS

Future studies on the aromatic amino acid hydroxylases will focus on the fine structure and function of this family of enzymes. X-ray crystallographic studies have been hampered by difficulties in the purification of these enzymes; the same properties which limit purification probably inhibit crystallization.

Important structure/function questions that need to be addressed in the future are the following: how does the regulatory domain mediate its inhibitory effect on enzyme activity, and what are the similarities/differences in the mechanisms of allosteric regulation in all three hydroxylases? Further delineation of the residues that are involved in pterin binding, iron binding and in particularly substrate specificity are required. This will give a clearer picture of the enzyme active site which will in turn facilitate a greater understanding of the enzyme mechanism. The role of multimerization and its mechanism needs to be resolved as this will provide an insight into enzyme regulation. It is clear that this group of enzymes still present a challenge even after 30 years of intense study.

REFERENCES

1 Kaufman, S. (1971) Adv. Enzymol. **35**, 245–319
2 Shiman, R. (1985) in Folates and Pterins (Blakely, R. L. and Benkovic, S. J., eds.), volume 2, pp. 179–249, John Wiley & Sons, New York
3 Kaufman, S. and Kaufman, E. E. (1985) in Folates and Pterins (Blakely, R. L. and Benkovic, S. J., eds.), volume 2, pp. 251–352, John Wiley & Sons, New York
4 Zigmond, R. E., Schwarzschild, M. A. and Rittenhouse, A. R. (1989) Annu. Rev. Neurosci. **12**, 415–461
5 Joh, T. H., Hwang, O. and Abate, C. (1986) in Neuromethods Series I (Boulton, A. A., Baker, G. B. and Yu, P. Y., eds.), pp. 1–32, Humana Press, Clifton, NJ
6 Boadle-Biber, M. C. (1993) Prog. Biophys. Mol. Biol. **60**, 1–15
7 Eisensmith, R. C. and Woo, S. L. C. (1991) Mol. Biol. Med. **8**, 3–18
8 Mason, S. T. (1984) Catecholamines and Behaviour, Cambridge University Press, Cambridge
9 Patel, K. P. and Schmid, P. G. (1986) in Brain Peptides and Catecholamines in Cardiovascular Regulation (Buckley, J. P. and Ferrano, C. M., eds.), pp. 53–64, Raven Press, New York
10 Pryer, J. C. and Sulser, F. (1991) in Biological Aspects of Affective Disorders (Horton, R. W. and Katona, C. L. E., eds.), pp. 77–94, Academic Press, New York
11 Grahame-Smith, D. G. (1992) in Serotonin 1A Receptors in Depression and Anxiety (Stahl, S. M., ed.), pp. 1–22, Raven Press, New York
12 Rao, D. N. and Kaufman, S. (1986) J. Biol. Chem. **261**, 8866–8876
13 Dahl, H.-H. M. and Mercer, J. F. B. (1986) J. Biol. Chem. **261**, 4148–4153
14 Scriver, C. R., Kaufman, S. and Woo, S. L. C. (1988) Annu. Rev. Genet. **22**, 301–321
15 Askin, D., Green, A. K., Dickson, A. J. and Fisher, M. J. (1990) Int. J. Biochem. **22**, 107–114
16 Baker, R. E., Jefferson, L. S. and Shiman, R. (1981) J. Cell Biol. **90**, 145–152
17 Kaufman, S. and Fisher, D. B. (1970) J. Biol. Chem. **245**, 4751–4759
18 Gillam, S. S., Woo, S. L. C. and Woolf, L. I. (1974) Biochem. J. **139**, 731–739
19 Nakata, H. and Fujisawa, H. (1980) Biochim. Biophys. Acta **614**, 313–327
20 Shiman, R., Gray, D. W. and Pater, A. (1979) J. Biol. Chem. **254**, 11300–11306
21 Woo, S. L. C., Gillam, S. S. and Woolf, L. I. (1974) Biochem. J. **139**, 741–749
22 Abita, J.-P., Blandon-Savoya, F. and Rey, F. (1987) Methods Enzymol. **142**, 27–35
23 Cotton, R. G. H. (1971) Biochim. Biophys. Acta **235**, 61–72
24 Richardson, S.-C. B. and Fisher, M. J. (1993) Int. J. Biochem. **25**, 581–588
25 Potter, L. T., Cooper, T., William, V. L. and Woolf, D. E. (1965) Circ. Res. **16**, 468–481
26 Kuczenski, R. T. and Mandell, A. J. (1972) J. Biol. Chem. **247**, 3114–3122
27 Haavik, J., Andersson, K. K., Petersson, L. and Flatmark, T. (1988) Biochim. Biophys. Acta **953**, 142–156
28 Andersson, K. K., Vassort, C., Brennan, B. A., Que, L., Flatmark, T., Haavik, J., Gros, F. and Thibault, J. (1992) Biochem. J. **284**, 687–695
29 Fitzpatrick, P. F., Chlumsky, L. J., Daubner, S. C. and O'Malley, K. L. (1990) J. Biol. Chem. **265**, 2042–2047
30 Daubner, S. C., Lauriano, C., Haycock, J. W. and Fitzpatrick, P. F. (1992) J. Biol. Chem. **267**, 12639–12646
31 Lovenberg, W., Jequier, E. and Sjoerdsma, A. (1967) Science **155**, 217–219
32 Hakanson, R., Lombard Des Gouttes, M. N. and Owman, C. (1967) Life Sci. **6**, 2577–2585
33 Gershon, M. D. (1981) Annu. Rev. Neurosci. **4**, 227–272
34 Kuhn, D. M. and Lovenberg, W. (1985) in Chemistry and Biochemistry of Pterins (Blakey, R. L. and Benkovic, S. J., eds.), pp. 353–382, Wiley & Sons, New York
35 Lovenberg, W., Besselaar, G. H., Bessinger, R. E. and Jacksons, R. L. (1973) in Serotonin and Behavior (Barchas, J. and Usdin, E., eds.), pp. 49–54, Academic Press, New York
36 Tong, J. H. and Kaufman, S. (1975) J. Biol. Chem. **250**, 4152–4158
37 Cash, C. D., Vazer, P., Mandel, P. and Maitre, M. (1985) Eur. J. Biochem. **149**, 239–245
38 Fujisawa, H. and Nakata, H. (1987) Methods Enzymol. **142**, 83–87
39 Kwok, S. C. M., Ledley, F. D., DiLella. A. G., Robson, K. J. H. and Woo, S. L. C. (1985) Biochemistry **24**, 556–561
40 Ledley, F. D., Grennet, H. E., DiLella, A. G., Kwok, S. C. M. and Woo, S. L. C. (1985) Science **228**, 77–79
41 DiLella. A. G., Marvit, J., Lidsky, A. S., Guttler, F. and Woo, S. L. C. (1986) Nature (London) **322**, 799–803
42 Eisensmith, R. C. and Woo, S. L. C. (1992) Hum. Mutat. **1**, 13–23
43 Ledley, F. D., Grennet, H. E., Dunbar, B. S. and Woo, S. L. C. (1990) Biochem. J. **267**, 399–406
44 Morales, G., Requena, J. M., Jimenez-Ruiz, A., Lopez, M. C., Ugarte, M. and Alonso, C. (1990) Gene **93**, 213–219
45 Lidsky, A. S., Law, M. L., Morse, H. G., Kao, F. T., Rabin, M., Ruddle, F. H. and Woo, S. L. C. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6221–6225
46 DiLella, A. G., Kwok, S. C. M., Ledley, F. D., Marvit, J. and Woo, S. L. C. (1986) Biochemistry **25**, 743–749
47 Grima, B., Lamaroux, A., Blanot, F., Biguet, N. F. and Mallet, J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 617–621
48 Grima, B., Lamaroux, A., Boni, C., Julien, J. F., Javoy-Agid, F. and Mallet, J. (1987) Nature (London) **326**, 707–711
49 Saadat, S., Stehle, A. D., Lamouroux, A., Mallet, J. and Thoenen, H. (1988) J. Neurochem. **51**, 572–578
50 Fauquet, M., Grima, B., Lamaroux, A. and Mallet, J. (1988) J. Neurochem. **50**, 142–148
51 Grenett, H. E., Ledley, F. D., Reed, L. L. and Woo, S. L. C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 5530–5534
52 Darmon, M. C., Guibert, B., Leviel, V., Ehret, M., Maitre, M. and Mallet, J. (1988) J. Neurochem. **51**, 312–316
53 Boularand, S., Darmon, M. C., Ganen, Y., Launay, J.-M. and Mallet, J. (1990) Nucleic Acids Res. **18**, 4257–4258
54 Stoll, J., Kozak, C. A. and Goldmann, D. (1990) Genomics **7**, 88–96
55 Craig, S. P., Buckel, V. J., Lamouroux, A., Mallet, J. and Craig, I. (1986) Cytogenet. Cell Genet. **42**, 29–32
56 Ledley, F. D., Grennet, H. E., Bartos, D. P., Van Tuinen, P., Ledbetter, D. H. and Woo, S. L. C. (1987) Somat. Cell Mol. Genet. **13**, 575–580
57 O'Malley, K. L. and Rotwein, P. (1988) Nucleic Acids Res. **16**, 4437–4446
58 Craig, S. P., Boularand, S., Darmon, M. C., Mallet, J. and Craig, I. W. (1991) Cytogenet. Cell Genet. **56**, 157–159
59 Lazarus, R. A., Dietrich, R. F., Wallick, D. E. and Benkovic, S. J. (1981) Biochemistry **20**, 6834–6841
60 Lazarus, R. A., Wallick, D. E., Dietrich, R. F., Gottshall, D. W., Benkovic, S. J., Gaffney, B. J. and Shiman, R. (1982) Fed. Proc. Fed. Am. Soc. Exp. Biol. **41**, 2605–2607
61 Dix, T. A., Kuhn, D. M. and Benkovic, S. J. (1987) Biochemistry **26**, 3354–3361
62 Haavik, J. and Flatmark, T. (1987) Eur. J. Biochem. **168**, 21–26
63 Dix, T. A., Bollag, G. E., Domanico, P. L. and Benkovic, S. J. (1985) Biochemistry **24**, 2955–2958
64 Siegmund, H.-U. and Kaufman, S. (1991) J. Biol. Chem. **266**, 2903–2910
65 Fisher, D. B., Kirkwood, R. and Kaufman, S. (1972) J. Biol. Chem. **247**, 5161–5167
66 Marota, J. J. A. and Shiman, R. (1984) Biochemistry **23**, 1303–1311
67 Shiman, R., Gray, D. W. and Hill, M. A (1994) J. Biol. Chem. **269**, 24637–24646
68 Wallick, D. E., Bloom, L. M., Gaffney, B. J. and Benkovic, S. J. (1984) Biochemistry **23**, 1295–1302
69 Bloom, L., Benkovic, S. J. and Gaffney, B. J. (1986) Biochemistry **25**, 4204–4210
70 Hill, M. A., Marota, J. J. A. and Shiman, R (1988) J. Biol. Chem. **263**, 5646–5655
71 Benkovic, S. J., Wallick, D., Bloom, L., Gaffney, B. J., Domanico, P., Dix, T. and Pember, S. (1985) Biochem. Soc. Trans. **13**, 436–438

72 Martinez, A., Abeygunawardana, C., Haavij, J., Klatmark, T. and Mildvan, A. S. (1993) Biochemistry **32**, 6381–6390
73 Dix, T. A. and Benkovic, S. J. (1988) Acc. Chem. Res. **21**, 101–107
74 Davis, M. D. and Kaufman, S. (1989) J. Biol. Chem. **264**, 8585–8596
75 Fitzpatrick, P. F. (1991) Biochemistry **30**, 3658–3662
76 Fitzpatrick, P. F. (1991) Biochemistry **30**, 6386–6391
77 Fitzpatrick, P. F. (1993) Adv. Exp. Med. Biol. **338**, 81–86
78 Kaufman, S. (1993) Adv. Enzymol. Relat. Areas Mol. Biol. **67**, 77–264
79 Ledley, F. D., DiLella, A. G., Kwok, S. C. M. and Woo, S. L. C. (1985) Biochemistry **24**, 3389–3394
80 Jennings, I. G., Russell, R. G. M., Armarego, W. L. F. and Cotton, R. G. H (1986) Biochem. J. **235**, 133–138
81 Cotton, R. G. H., McAdam, W., Jennings, I. and Morgan, K. J. (1988) Biochem. J. **255**, 193–196
82 Zhao, G., Xia, T., Song, J. and Jensen, R. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1366–1370
83 Onishi, A., Liotta, L. J. and Benkovic, S. J. (1991) J. Biol. Chem. **266**, 18454–18459
84 Neckameyer, W. S. and White, K. (1992) J. Biol. Chem. **267**, 4199–4206
85 Brown, E. R., Coker, G. T and O'Malley, K. L. (1987) Biochemistry **26**, 5208–5212
86 Celikel, R., Davis, M. D., Dai, X., Kaufman, S. and Xuong, N.-H. (1991) J. Mol. Biol. **218**, 495–498
87 Markey, K. A., Kondo, S., Shenkman, L. and Goldstein, M. (1980) Mol. Pharmacol. **17**, 79–84
88 Nagatsu, T. and Ichinose, H. (1991) Comp. Biochem. Physiol. **98**, 203–210
89 Nakata, H. and Fujisawa, H. (1982) Eur. J. Biochem. **124**, 595–601
90 Kaufman, S. and Fisher, D. B. (1970) J. Biol. Chem. **245**, 4745–4750
91 Iwaki, M., Phillips, R. S. and Kaufman, S. (1986) J. Biol. Chem. **261**, 2051–2056
92 Parniak, M. A. (1987) in Unconjugated Pterins and Related Biogenic Amines (Curtius, H. Ch., Ghisla, S., Blau, N. and Levine, R. A., eds.), pp. 327–337, Walter de Gruyter, Berlin, New York
93 Liu, X. and Vrana, K. (1991) Neurochem. Int. **18**, 27–31
94 Lohse, D. L. and Fitzpatrick, P. F. (1993) Biochem. Biophys. Res. Commun. **197**, 1543–1548
95 Smith, S. C., Kemp, B. E., McAdam, W. J., Mercer, J. F. B. and Cotton, R. G. H. (1984) J. Biol. Chem. **259**, 11284–11289
96 Mercer, J. F. B., McAdam, W., Chambers, G. W. and Walker, I. D. (1986) Biochem. J. **236**, 679–683
97 Kanada, N., Kobayashi, K., Ichinose, H., Kishi, F., Nagazawa, A., Kurosawa, Y., Fujita, K. and Nagatsu, T. (1987) Biochem. Biophys. Res. Commun. **146**, 971–975
98 O'Malley, K. L., Anhalt, M. J., Martin, B. M., Kelsoe, J. R., Winfield, S. L. and Ginns, E. I. (1987) Biochemistry **26**, 6910–6914
99 Haycock, J. W. (1991) Anal. Biochem. **181**, 259–266
100 Meek, J. L. and Neff, N. H. (1972) J. Neurochem. **19**, 1519–1525
101 Lovenberg, W. and Bruckwick, E. (1977) in Structure and Function of Monoamine Enzymes (Usdin, E., Weiner, N. and Youdin, M. B. H., eds.), pp. 43–58, Dekker, New York
102 Kim, K. S., Wessel, T. C., Stone, D. M., Carver, C. H., Joh, T. H. and Park, D. H. (1991) Mol. Brain Res. **9**, 277–283
103 Dumas, S., Darmon, M. C., Delort, J. and Mallet, J. (1989) J. Neurosci. Res. **24**, 537–547
104 Gibbs, B. S. and Benkovic, S. J. (1991) Biochemistry **30**, 6795–6802
105 Cox, D. D., Benkovic, S. J., Bloom, L. M., Bradley, F. C., Nelson, M. J., Que, L., Jr. and Wallick, D. E. (1988) J. Am. Chem. Soc. **110**, 2026–2032
106 Daubner, S. C. and Fitzpatrick, P. F. (1993) Arch. Biochem. Biophys. **302**, 455–460
107 Cotton, R. G. H and Jennings, I. G. (1978) Eur. J. Biochem. **85**, 357–363
108 Cotton, R. G. H. and Gratten, P. J. (1975) Eur. J. Biochem. **60**, 427–430
109 Jennings, I. G., Kemp, B. E. and Cotton, R. G. H. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5734–5738
110 Jennings, I. G. and Cotton, R. G. H. (1990) J. Biol. Chem **265**, 1885–1889
111 Dickson, P. W., Jennings, I. G. and Cotton, R. G. H. (1994) J. Biol. Chem. **269**, 20369–20375
112 Gottschall, D. W., Deitrich, R. F., Benkovich, S. J. and Shiman, R. (1982) J. Biol. Chem. **257**, 845–849
113 Gibbs, B. S., Wojchowski, D. and Benkovic, S. J. (1993) J. Biol. Chem. **268**, 8046–8052
114 Haavik, J., Le Bourdelles, B., Martinez, A., Flatmark, T. and Mallet, J. (1991) Eur. J. Biochem. **199**, 371–378
115 Kuhn, D. M., Ruskin, B. and Lovenberg, W. (1980) J. Biol. Chem. **255**, 4137–4143
116 Bloom, L. M., Benkovic, S. J. and Gaffney, B. J. (1986) Biochemistry **25**, 4204–4210
117 Martinez, A., Haavik, J. and Flatmark, T. (1990) Eur. J. Biochem. **193**, 211–219
118 Martinez, A., Andersson, K. K., Haavik, J. and Flatmark, T. (1991) Eur. J. Biochem. **198**, 675–682
119 Martinez. A., Olafsdottir, S. and Flatmark, T. (1993) Eur. J. Biochem. **211**, 259–266
120 Haavik, J., Martinez, A. and Flatmark, T. (1990) FEBS Lett. **262**, 363–365
121 Almas, G., le Bourdelles, B., Flatmark, T., Mallet, J. and Haavik, J. (1992) Eur. J. Biochem. **209**, 249–255
122 Pember, S. O., Villafranca, J. J. and Benkovic, S. J. (1986) Biochemistry **25**, 6611–6619
123 Carr, R. T. and Benkovic, S. J. (1993) Biochemistry **32**, 14132–14138
124 Kaufman, S. and Mason, K. (1982) J. Biol. Chem. **257**, 14667–14678
125 Parniak, M. A. and Kaufman, S. (1981) J. Biol. Chem. **256**, 6876–6882
126 Tourian, A. (1971) Biochim. Biophys. Acta **242**, 345–354
127 Ayling, J. E. and Helfand, G. D. (1975) in Chemistry and Biology of Pteridines (Pfeiderer, W., ed.), pp. 305–319, Walter de Gruyter, Berlin
128 Shiman, R. (1980) J. Biol. Chem. **255**, 10029–10032
129 Shiman, R., Jones, S. H. and Gray, D. W. (1990) J. Biol. Chem. **265**, 11633–11642
130 Caillaud, C., Lyonnet, S., Rey, F., Melle, D., Frebourg, T., Berthelon, M., Vilarinho, L., Vaz Osorio, R., Rey, J. and Munnich, A. (1991) J. Biol. Chem. **266**, 9351–9354
131 Kataoka, K., Takada, H., Yoshimura, T., Furuyoshi, S., Esaki, N., Ohshima, T. and Soda, K. (1993) J. Biochem. (Tokyo) **114**, 69–75
132 Fisher, D. B. and Kaufman, S. (1973) J. Biol. Chem. **248**, 4345–4353
133 Shiman, R. and Gray, D. W. (1980) J. Biol. Chem. **255**, 4793–4800
134 Parniak, M. A., Davis, M. D. and Kaufman, S. (1988) J. Biol. Chem. **263**, 1223–1230
135 Kuczenski, R. (1973) J. Biol. Chem. **248**, 2261–2265
136 Lloyd, T. and Kaufman, S. (1974) Biochem. Biophys. Res. Commun. **59**, 1262–1269
137 Raese, J., Patrick, R. L. and Barchas, J. D. (1976) Biochem. Pharmacol. **25**, 2245–2250
138 Hamon, P., Burgoin, S., Artaud, H. and Hery, F. (1977) J. Neurochem. **28**, 811–818
139 Hamon, M., Bourgoin, S., Hery, F., Ternaux, J. P. and Glowinski, J. (1976) Nature (London) **260**, 61–63
140 Hamon, M., Burgoin, S., Hery, F. and Simonnet, G. (1978) Biochem. Pharmacol. **27**, 915–922
141 Petrack, B., Sheppy, F. and Fetzer, V. (1968) J. Biol. Chem. **243**, 743–748
142 Daubner, S. C., Lohse, D. L. and Fitzpatrick, P. F. (1993) Protein Sci. **2**, 1452–1460
143 Walker, S. J., Liu, X., Roskoski, R. and Vrana, K. E. (1994) Biochim. Biophys. Acta **1206**, 113–119
144 Yang, X. and Kaufman, S. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 6659–6663
145 Katz, I. R., Yamauchi, T. T. and Kaufman, S. (1976) Biochim. Biophys. Acta **429**, 84–95
146 Gahn, L. G. and Roskoski, R., Jr. (1993) Biochem. J. **295**, 189–194
147 Abita, J.-P., Milstein. S., Chang, N. and Kaufman, S. (1976) J. Biol. Chem. **251**, 5310–5314
148 Doskeland, A. P., Schworer, C. M., Doskeland, S. O., Chrisman, T. D., Soderling, T. R., Corbin, J. D. and Flatmark, T. (1984) Eur. J. Biochem. **145**, 31–37
149 Donlan, J. and Kaufman, S. (1978) J. Biol. Chem. **253**, 6657–6659
150 Smith, S. C., McAdam, W. J., Kemp, B. E., Morgan, F. J. and Cotton, R. G. H. (1987) Biochem. J. **244**, 625–631
151 Green, A. K., Cotton, R. G. H., Jennings, I. G. and Fisher, M. J. (1990) Biochem. J. **265**, 563–568
152 Richardson, S.-C. B., Aspbury, R. A. and Fisher, M. J. (1993) Biochem. J. **292**, 419–424
153 Nairn, A. C., Hemmings, H. C., Jr. and Greengard, P. (1985) Annu. Rev. Biochem. **54**, 931–976
154 Kuhn, D. M., O'Callaghan, J. P., Juskevich, J. and Lovenberg, W. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 4688–4691
155 Ehret, M., Cash, C. D., Harmon, M. and Maitre, M. C. (1989) J. Neurochem. **52**, 1886–1891
156 Boadle-Biber, M. C. (1980) Biochem. Pharmacol. **29**, 669–672
157 Makita, Y., Akuno, S. and Fujisawa, H. (1990) FEBS Lett. **268**, 185–188
158 Yamauchi, T., Nakata, H. and Fujisawa, H. (1981) J. Biol. Chem. **256**, 5404–5409
159 Ichimura, T., Isobe, T., Okyama, T., Taahashi, N., Araki, K., Kuwano, R. and Takashi, Y. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7084–7088
160 Isobe, T., Ichimura, T., Sunaya, T., Okuyama, T., Takahashi, N., Kuwano, R. and Takahashi, Y. (1991) J. Mol. Biol. **217**, 125–132
161 Milstein, S., Abita, J.-P., Chang, N. and Kaufman, S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 1591–1593
162 Shiman, R., Mortimore, G. E., Schworer, C. M. and Gray, D. W. (1982) J. Biol. Chem. **257**, 11213–11216
163 Doskeland, A. P., Doskeland, S. O., Ogreid, D. and Flatmark, T. (1984) J. Biol. Chem. **259**, 11242–11248
164 Pollock, R. J., Kapatos, G. and Kaufman, S. (1981) J. Neurochem. **37**, 855–860
165 Kuhn, D. M., Vogel, R. L. and Lovenberg, W. (1978) Biochem. Biophys. Res. Commun. **59**, 1262–1269

166 Yamauchi, T. and Fujisawa, H. (1979) Arch. Biochem. Biophys. **198,** 219–226
167 Wretborn, M., Humble, E., Ragnarsson, U. and Engstrom. L. (1980) Biochem. Biophys. Res. Commun. **93** 403–408
168 Doskeland, A. P., Vintermyr, O. K., Flatmark, T., Cotton, R. G. H. and Doskeland, S. O. (1992) Eur. J. Biochem. **206**, 161–170
169 Kemp, B. E. and Pearson, R. B. (1990) Trends Biochem. Sci. **15**, 342–346
170 Kemp, B. E. (1979) J. Biol. Chem. **254**, 2638–2642
171 Citron, B. A., Davis, M. D. and Kaufman, S. (1994) Biochem. Biophys. Res. Commun. **198**, 174–180
172 Campbell, D. G., Hardie, D. G. and Vulliet, P. R. (1986) J. Biol. Chem. **261**, 10489–10492
173 Haycock, J. W. (1990) J. Biol. Chem. **265**, 11682–11691
174 Haycock, J. W. and Haycock, D. A. (1991) J. Biol. Chem. **266**, 5650–5657
175 Haycock, J. W. (1993) Neurochem. Res. **18**, 15–26
176 Vulliet, P. R., Woodgett, J. R. and Cohen, P. (1984) J. Biol. Chem. **259**, 13680–13683
177 Albert, K. A., Helmer-Matyjek, E., Nairn, A. C., Miller, T. H., Haycock, J. W., Green, L. A., Goldstein, M. and Greengard, P. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 7713–7717
178 Vulliet, P. R., Woodgett, J. G., Ferrari, S. and Hardie, D. G. (1985) FEBS Lett. **182**, 335–339
179 Atkinson, J., Richland, N., Scworer, C., Kuczenski, R. and Soderling, T. (1987) J. Neurochem. **49**, 1241–1248
180 Haycock, J. W., Ahn, N. G., Cobbs, M. H. and Krebs, E. G. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 2365–2369
181 Wu, J., Filer, D., Friedhoff, A. J. and Goldstein, M. (1992) J. Biol. Chem. **267**, 25754–25758
182 Daubner, S. C., Lauriano, C., Haycock, J. W. and Fitzpatrick, P. F. (1992) J. Biol. Chem. **267**, 12639–12646
183 Pearson, R. B., Woodgett, J. R., Cohen, P. and Kemp, B. E. (1985) J. Biol. Chem. **260**, 8465–8473
184 Nagatsu, T., Sawada, M. and Yamaguchi,T. (1983) Neurochem. Int. **5**, 603–609
185 Jedlicki, E., Kaufman, S. and Milstein, S. (1977) J. Biol. Chem. **252**, 7711–7714
186 Pelech, S., Cohen, P., Fisher, M. J., Pogson, C. I., El-Maghrabi, M. R. and Pilkis, S. J. (1984) Eur. J. Biochem. **145**, 39–49
187 Alemany, S., Tung, H. Y. L., Shenoliker, S., Pilkis, S. J. and Cohen, P. (1984) Eur. J. Biochem. **145**, 51–56
188 Haavik, J., Schelling, D. L., Andersson, K. K., Campbell, D. G., Flatmark, T. and Cohen, P. (1989) FEBS Lett. **251**, 36–42
189 Phillips, R. S., Parniak, M. A. and Kaufman, S. (1984) Biochemistry **23**, 3836–3842
190 Tipper, J. and Kaufman, S. (1992) J. Biol. Chem. **267**, 889–896
191 Phillips, R. S. and Kaufman, S. (1984) J. Biol. Chem. **259**, 2474–2479
192 Xia, T., Gray, D. W. and Shiman, R. (1994) J. Biol. Chem. **269**, 24657–24665
193 Shiman, R., Xia, T., Hill, M. A. and Gray, D. W. (1994) J. Biol. Chem. **269**, 24647–24656
194 Parniak, M. A. (1990) in Chemistry and Biology of Pteridines (Curtius, H.-Ch. and Blau, N., eds.), pp. 660–663, Walter de Gruyter, Berlin
195 Parniak, M. J., Jennings, I. G. and Cotton, R. G. H. (1989) Biochem. J. **257**, 383–388
196 Andersson, K. K., Cox, D. D., Que, L., Flatmark, T. and Haavik, J. (1988) J. Biol. Chem. **263**, 18621–18626
197 Haavik, J., Martinez, A. and Flatmark, T. (1990) FEBS Lett. **262**, 363–365
198 Miller, M. R and Shiman, R. (1976) J. Biol. Chem. **251**, 3671–3676
199 Bokkino, S. B., Blackmore, P. F. and Exton, J. H. (1985) J. Biol. Chem. **260**, 14201–14207
200 Wang, Y., Citron, B. A., Ribeiro, P. and Kaufman, S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8779–8783
201 Kiuchi, K., Titani, K., Fujita, K., Suzuki, K. and Nagatsu, T. (1991) Biochemistry **30**, 10416–10419
202 Shiman, R., Akino, M. and Kaufman, S. (1971) J. Biol. Chem. **246**, 1330–1340
203 Katz, I., Lioyd, T. and Kaufman, S. (1976) Biochim. Biophys. Acta **445**, 567–578
204 Ribeiro, P., Pigeon, D. and Kaufman, S. (1991) J. Biol. Chem. **266**, 16207–16211
205 Fitzpatrick, P. F., Chlumsky, L. J., Daubner, S. C. and O'Malley, K. L. (1990) J. Biol. Chem. **265**, 2042–2047
206 Haavik, J., Doskeland, A. and Flatmark, T. (1986) Eur. J. Biochem. **160**, 1–8
207 Koizumi, S., Tanaka, F., Kaneda, N., Kano, K. and Nagatsu, T. (1988) Biochemistry **27**, 640–646
208 Martinez, A., Andersson, K. K., Dahle, G. and Flatmark, T. (1989) Pteridines and Folic Acid Derivatives (Curtius, H.-Ch., Ghisla, S. and Blau, N., eds.), pp. 644–647, Walter de Gruyter, New York
209 Phillips, R. S., Iwaki, M. and Kaufman, S. (1983) Biochem. Biophys. Res. Commun. **110**, 919–925
210 Harada, T., Kagamiyama, H. and Hatakeyama, K. (1993) Science **260**, 1507–1510
211 Davis, M. D., Ribeiro, P., Tipper, J. and Kaufman, S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10109–10113
212 Citron, B. A., Kaufman, S., Milstein, S., Naylor, E. W., Greene, C. L. and Davis, M. D. (1993) Am. J. Hum. Genet. **53**, 768–774
213 Koneki, D. S., Wang, Y., Trefz, F. K., Lichter-Koneki, U. and Woo, S. L. C. (1992) Biochemistry **31**, 8363–8368
214 Chiapelli, F., Haggerty, D. F., Lynch, M. and Popjak, G. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 2105–2109
215 Lewis, E. J., Harrington, C. A. and Chikaraishi, D. M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3550–3554
216 Stoll, J. and Goldman, D. (1991) J. Neurosci. Res. **28**, 457–465
217 Kaufman, S. (1970) J. Biol. Chem. **245**, 4751–4759
218 Lazarus, R. A., Benkovic, S. J. and Kaufman, S. (1983) J. Biol. Chem. **258**, 10960–10962
219 Citron, B. A., Davis, M. D., Milstein, S., Gutierrez, J., Mendel, D. B., Crabtree, G. R. and Kaufman, S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 11891–11894
220 Hauer, C. H., Rebrin, I., Thony, B., Neuheiser, F., Curtius, C. H., Hunziker, P., Blau, N., Ghisla, S. and Heizmann, C. W. (1993) J. Biol. Chem. **268**, 4828–4831
221 Mendel, D. M., Khaven, P. A., Conley, P. B., Graves, M. K., Hansen, L. P., Admon, A. and Crabtree, G. R. (1991) Science **254**, 1762–1767
222 Wang, Y., Hahn, T. M., Tsai, S. Y. and Woo, S. L. C. (1994) J. Biol. Chem. **269**, 9137–9146
223 Cotton, R. G. H., Howells, D. W., Saleeba, J. A., Dianzani, I., Smooker, P. M. and Jennings, I. G. (1993) in Chemistry and Biology of Pteridines and Folates (Ayling, J. E., Gopal Nair, M. and Baugh, C. M., eds.), pp. 55–57, Plenum Press, New York