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Homology between Phenylalanine and Tyrosine Hydroxylases Reveals Common Structural and Functional Domains†

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ABSTRACT: Phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TYH) are mixed-function oxidases that share many characteristic biochemical and immunological properties. The recent cloning and sequencing of full-length cDNAs for both human PAH and rat TYH allow detailed comparison of their primary structures. There is a high degree of homology between PAH and TYH on nucleic acid and amino acid levels. The pattern of homology suggests that these molecules are comprised of a homologous core containing the determinants for enzymatic activity and a nonhomologous region that contributes to substrate specificity and regulation. The degree of homology also suggests that these two proteins evolved from a common ancestor.

The aromatic amino acids phenylalanine, tyrosine, and tryptophan are each oxidized in higher eukaryotes by specific hydroxylases. Phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TYH), and tryptophan hydroxylase are specific enzymes having distinct substrate specificities and physical characteristics, yet these enzymes share many similarities in structure and function. All three enzymes are mixed-function oxidases that utilize molecular oxygen to oxidize simultaneously the aromatic amino acid substrates and a pterin cofactor, tetrahydrobiopterin (Kaufman & Fisher, 1974). Moreover, the mechanism of the hydroxylation reaction catalyzed by each enzyme is similar (Kaufman & Fisher, 1974). Under appropriate conditions, phenylalanine, tyrosine, and tryptophan can be used as alternative substrates or competitive inhibitors for the other hydroxylases (Kaufman & Fisher, 1974; Kaufman & Mason, 1982; Abita et al., 1984; Parniak & Kaufman, 1981). In addition, these proteins share common antigenic determinants (Kaufman & Fisher, 1974; Friedman et al., 1972; Chikaraishi et al., 1983). These observations have led to the frequent suggestion that these enzymes might be members of a family of structurally related proteins.

The recent cloning and sequencing of phenylalanine hydroxylase cDNA from rat and human liver (Robson et al., 1982, 1984; Woo et al., 1983; Kwok et al., 1985) and tyrosine hydroxylase cDNA from rat pheochromocytoma cell lines (Lamouroux et al., 1982; Chikaraishi et al., 1983; Grima et al., 1985) allow direct comparison of the primary structure of these enzymes for the first time. This analysis reveals extensive homology between the nucleic acid and amino acid sequences of the two enzymes. The observed homology between PAH and TYH accounts for many of the observed similarities in structure and function and suggests that these two proteins evolved from a common ancestor.

Materials and Methods

Nucleic Acid and Amino Acid Sequences. The human PAH sequence was determined from a full-length cDNA clone phPAH247 obtained from a human liver library (Kwok et al., 1985). This clone comprises 2428 bases excluding a 19-base poly(A) tail. There is a 5′ untranslated region of 222 bases, an open reading frame of 1353 bases, and a 3′ untranslated region of 851 bases. The open reading frame codes for an amino acid sequence of 452 amino acids. The predicted amino acid composition agrees with that determined experimentally for the rat enzyme, and the predicted amino acid sequence also matches a 17 amino acid sequence representing the amino terminus of a CNBr fragment of the purified rat enzyme (Robson et al., 1982; Kwok et al., 1985). This full-length clone has been utilized for genetic expression studies in eukaryotes (Ledley et al., 1985) and prokaryotes (Ledley, Grenett, and Woo, unpublished observations) and shown to direct the

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synthesis of PAH enzyme activity. These studies demonstrated that the cDNA clone encodes authentic PAH and contains all of the genetic information necessary for synthesis of the enzyme (Ledley et al., 1985).

The partial rat PAH sequences was determined for two overlapping PAH cDNA clones identified in a rat liver library (rPAH98 and rPAH91) that together represent 1203 bases from the 3′ end of the rat PAH cDNA. These clones hybrid selected mRNA coding for immunoreactive rat PAH, and the nucleic acid sequence matched fragments of the amino acid sequence of the purified enzyme (Robson et al., 1982, 1984).

The rat TYH sequence published by Grima et al. (1985) was derived from a full-length cDNA clone comprising 1770 bases excluding a poly(A) tail. This clone contains an open reading frame of 1494 bases coding for a protein of 498 amino acids. The authenticity of these clones was established by hybrid selection of mRNA coding for immunoreactive TYH (Lamouroux et al., 1982). Both rat TYH and bovine TYH were shown to have an AUG initiation codon at an identical position in the 5′ end of the cDNA, confirming the assignment of the open reading frame (Grima et al., 1985).

Computer Analysis. Analysis and alignment of protein sequences were performed with the on-line facilities of the Protein Identification Resource (PIR). Optimal alignments were determined with the program ALIGN (Barker & Dayhoff, 1972) based on the algorithm of Needleman & Wunsch (1970). Calculations were made by using the mutation data matrix (MDM) (Schwartz & Dayhoff, 1978; Dayhoff et al., 1972) with bias = 6 and penalty = 25. Statistical analysis was performed by comparing the optimal alignment score with the mean and standard deviation of 30 alignment scores calculated for randomized sequences having the identical amino acid composition as PAH and TYH (Dayhoff et al., 1983). Calculation of evolutionary distance between proteins in accepted point mutations (PAMs) was performed by using standard methods (Dayhoff et al., 1972; Dayhoff, 1976).

Nucleic acid comparisons were made by using a dot matrix program written for the IBM PC (Zweig, 1984). Analysis was performed using a window in which 11 or 12 consecutive bases were required to match.

Results

Alignment of the amino acid sequences of rat TYH, human PAH, and rat PAH reveals a striking degree of homology that is unevenly distributed in the molecules (Figure 1). This alignment matches 219 of 449 amino acids (48%) excluding gaps. There is little, if any, homology between the two proteins at the amino terminus where less than 20% of the first 150 amino acids match. Considerable homology is apparent in the central region and carboxyl terminus of the two proteins, particularly in the spacing of five shared cysteine residues (rat TYH residues 249, 263, 311, 330, and 380 and human PAH residues 203, 217, 265, 284, and 334) and the surrounding regions. For example, rat TYH residues 238–400 and human PAH residues 192–354 exhibit over 75% identity with no gaps. Furthermore, many of the substitutions shown in the alignment in Figure 1 are conservative (e.g., Phe and Tyr, Arg and Lys, Thr and Ser). Overall, the degree of homlogy between TYH and PAH is highly statistically significant (>50 standard deviations from the mean) when compared to random sequences with identical amino acid compositions.

There are, however, important differences between these two proteins. Most notable is the larger size of rat TYH, which contains about 50 additional amino acids at the amino terminus (Figure 1). There are also nonhomologous cysteine residues 177 and 329 in rat TYH and residues 29, 237, 357, and 445 in human PAH. These changes could cause substantial differences in the secondary and tertiary structure of these two molecules.

The evolutionary distance between these three peptide sequences was estimated for the regions represented in the incomplete rat PAH and both the human PAH and rat TYH clones. An evolutionary distance of 5 PAMs was calculated for the overlapping regions of rat PAH and human PAH sequences. Since rat and human ancestors are believed to have diverged approximately 75 million years ago (Dayhoff, 1978), this corresponds to a mutational rate of approximately 7 PAMs/100 million years. An evolutionary distance of 51–55 PAMs was calculated between rat or human PAH and rat TYH, considering only those regions overlapping the incomplete rat PAH sequence (PAH residues 208–452 and TYH residues 254–498).

Dot matrix comparisons of rat TYH and human PAH cDNA sequences also demonstrate a large degree of homology at the nucleic acid level (Figure 2). No homology is apparent in the extreme 5′ sequences of rat TYH and human PAH. There is considerable homology between the central regions of the genes; for example, nucleotides 632–1301 in rat TYH and nucleotides 796–1284 in human PAH (corresponding to the regions of maximal amino acid homology described above) exhibit over 60% homology with no gaps. It is significant that nucleic acid homology deteriorates at the same positions toward the 5′ end of the gene where amino acid homology is lost, since this indicates that the lack of homology at the amino terminus cannot be accounted for by a simple frameshift error in the sequencing of either cDNA clone. In addition, no homology is seen between the 3′ untranslated regions of these clones distal to the termination codons at 1576 for human PAH and 1506 for rat TYH.

Discussion

The extensive homology between TYH and PAH provides important insight into the structure and function of these enzymes. PAH from rat liver and TYH from rat pheochromocytoma cells or bovine adrenal have been characterized extensively and are known to share many common structural and functional characteristics. There is considerably less known about the biochemical properties of human PAH, although the limited data available suggest that the characteristics of the human enzyme are generally similar to those of the rat (Friedman & Kaufman, 1973; Woo et al., 1974).

Both PAH and TYH are mixed-function oxidases that contain binding sites of a reduced pterin cofactor, molecular oxygen, iron, and their respective substrates (Kaufman & Fisher, 1974). Both catalyze hydroxylation of an aromatic amino acid by a similar mechanism involving transient formation of a charged quaternary complex intermediate (Kaufman & Fisher, 1974). While PAH and TYH exhibit considerable specificity for phenylalanine and tyrosine, respectively, both amino acids can competitively inhibit heterologous enzyme activity and, under appropriate conditions, can serve as alternative substrates for hydroxylation (Kaufman & Fisher, 1974; Kaufman & Mason, 1982; Abita et al., 1984; Parniak & Kaufman, 1981). There are also many similarities in the regulation of PAH and TYH. The activity of both enzymes is increased by phosphorylation (Abita et al., 1976; Smith et al., 1984; Vulliet et al., 1984), partial chymotryptic digestion (Fisher & Kaufman, 1972, 1973; Vigny & Henry, 1981; Hoeldtke & Kaufman, 1977), and treatment with certain phospholipids (Fisher & Kaufman, 1972, 1973; Hoeldtke & Kaufman, 1977). There is also biochemical and immuno-

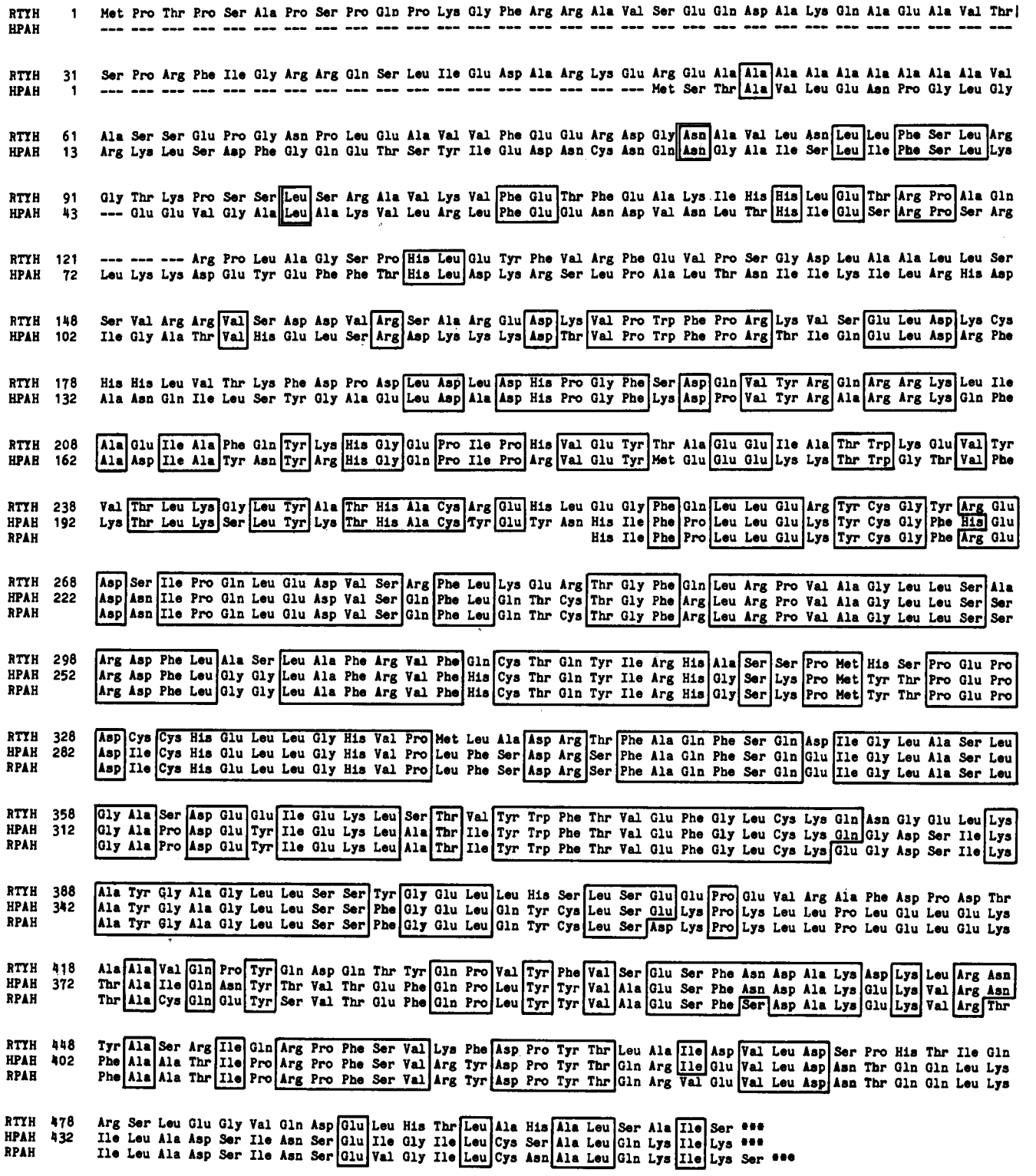

FIGURE 1: Alignment of protein sequences of rat tyrosine hydroxylase (RTYH) (Grima et al., 1985), human phenylalanine hydroxylase (HPAH) (Kwok et al., 1985), and a fragment of rat phenylalanine hydroxylase (RPAH) (Robson et al., 1984). Amino acid numbers for each sequence are shown at the left. Alignment is based on the optimal alignment determined by the program ALIGN (Barker & Dayhoff, 1972). Identical residues between TYH and either PAH are enclosed in boxes.

logical evidence of considerable structural similarity between PAH and TYH. Antibodies raised against rat PAH cross-react with TYH (Friedman et al., 1972), and rat PAH cDNA clones hybridize to mRNAs in rat adrenal that most likely represent TYH (Robson et al., 1982). The degree of homology between rat TYH and human PAH is entirely consistent with the broad similarities in structure and function.

It is reasonable to predict that the common structures and activities of TYH and PAH are encoded by the homologous regions of the proteins. On the other hand, not all of the common characteristics of PAH and TYH can be accounted for by this homology. For example, although both TYH and PAH are phosphoproteins that are substrates for the cAMP-dependent protein kinase (Abita et al., 1976; Wretborn et al., 1980; Smith et al., 1984; Vulliet et al., 1984), there is no apparent homology between the sequences of the phosphorylation sites in PAH and TYR. The phosphorylation site of rat PAH has been determined experimentally (Wretborn et

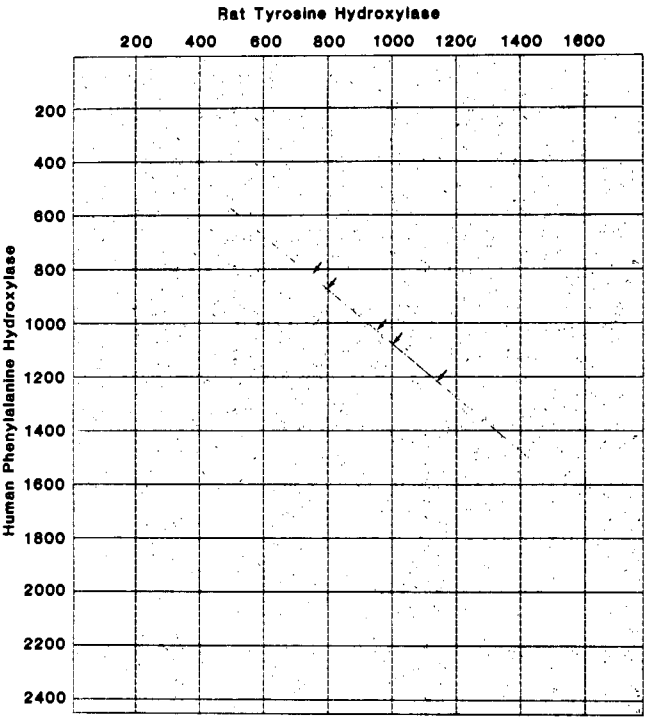

FIGURE 2: Dot matrix comparison of nucleic acid sequences of rat TYH cDNA (Grima et al., 1985) and human PAH cDNA (Kwok et al., 1975). Dots indicate homologous windows in which 11 of 12 consecutive residues are identical in both sequences. Arrows indicate the position of codons for cysteine residues that are common to both sequences as shown in the alignment of amino acid sequences in Figure 1.

al., 1980) and is identical with a region of human PAH at positions 13–21 surrounding the Ser residue at position 16 (Kwok et al., 1985). This sequence of the PAH phosphorylation site shares limited homology with rat TYH sequences surrounding Ser-40, which appears to contain a consensus sequence characteristic of cAMP-dependent phosphorylation sites (Grima et al., 1985).

Proteolytic cleavage has been studied as a means of activating both PAH and TYH and dissecting the functional regions of both molecules (Kaufman & Fisher, 1974; Abita et al., 1984; Fisher & Kaufman, 1972, 1973; Vigny & Henry, 1981; Hoeldtke & Kaufman, 1977). Partial proteolytic digestion of either enzyme with chymotrypsin preserves catalytic activity and also "stimulates" enzyme activity by altering kinetic properties toward the preferred substrate. Partial proteolysis also disproportionally increases the relative enzymatic activity toward the alternative substrates (Kaufman & Fisher, 1974; Abita et al., 1984). The active proteolytic fragment of PAH is known to have a molecular weight of approximately 35 000 and does not contain the phosphorylated site (Abita et al., 1976; Fisher & Kaufman, 1972). Given the sequence of PAH and that the phosphorylation site is near the amino terminus, it can be inferred that the active fragment most probably encompasses the carboxyl end of the complete protein (Kwok et al., 1985). The active proteolytic fragment of TYR is estimated to be 34 000 daltons (Vigny & Henry, 1981; Hoeldtke & Kaufman, 1977) and is also believed to include the carboxyl end of the protein on the basis of comparisons of biochemical and sequence data (Grima et al., 1985).

While the exact sites of proteolytic cleavage have not been determined for either protein, the approximate regions in which the cleavage may occur can be estimated by comparing the size of the proteolytic fragments with the amino acid sequences. The last 305 amino acids of human PAH (amino acids 148–452) constitute a peptide of 35 070 daltons, and the last 299 amino acids of rat TYH (amino acids 226–498) constitute a peptide for 34 020 daltons. It is apparent from the alignment in Figure 1 that cleavage in these general regions would separate the amino terminus, in which there is little amino acid homology, from the remainder of the proteins, which contain most of the homologous sequences. This is consistent with the hypothesis that the highly homologous sequences represent the common determinants for enzymatic activity. In addition, the increase in relative homology between the two active proteolytic fragments parallels the loss of substrate specificity.

This observation also suggests a structural correlation for the hypothesis that regulation of PAH and TYH activity involves modulation of an "inhibitory" region. This thesis suggests that normal regulation of PAH activity involves suppression of the active site by an inhibitory peptide that can be irreversibly removed by protease digestion. This inhibitory regulation of PAH is reversibly regulated by treatment of the enzyme with protein kinase (Abita et al., 1976), lysolecithin (Fisher & Kaufman, 1972, 1973), or the sulfhydryl modifying agent *N*-ethylmaleimide (Parniak & Kaufman, 1981). The intrinsic inhibitory function of TYH is similarly regulated by proteolysis (Vigny & Henry, 1981; Hoeldtke & Kaufman, 1977), phosphorylation (Hoeldtke & Kaufman, 1977), and phospholipids (Hoeldtke & Kaufman, 1977). After digestion of PAH and TYH with chymotrypsin, however, neither phosphorylation nor lysolecithin has any stimulatory effect. The sequences and pattern of homology between TYH and PAH suggest that the nonhomologous regions at the amino terminus of the proteins may represent the postulated inhibitory peptide that is removed from the active catalytic cores by proteolysis. The phosphorylation site of PAH is known to be within this region, and it is reasonable to speculate that the effective sites of lysolecithin and phosphorylation in TYH will be found in this region as well.

Thus the pattern of homology between TYH and PAH is entirely consistent with experimental data concerning the structure and function of the enzymes. These data suggest that these enzymes contain two distinct domains with different functions. The first is a highly homologous domain at the center and carboxyl terminus of each molecule, representing the active core of the enzyme. This domain contains all of the structural determinants required for the hydroxylation reaction, and while it exhibits significant substrate specificity, it is relatively promiscuous compared to the complete enzyme. The second is a nonhomologous domain at the amino terminus of the molecules representing regulatory determinants that contribute to substrate specificity and regulation of the two enzymes. This model of two distinct functional regions can be tested by site-directed mutagenesis of the cloned TYH and PAH genes.

The extensive homology between TYH and PAH also indicates that these two proteins evolved from a common ancestoral gene. Statistical analysis of the degree of homology between these two proteins, compared to the degree of homology expected by random change between proteins with identical amino acid compositions, indicates that it is exceedingly improbable that this amount of homology arose by chance. Rather, it is likely that the gene for a common ancestoral protein underwent duplication into separate genetic loci, which subsequently evolved specialized functions. A rough estimate of when this duplication may have occurred can be calculated by comparing the rate of divergence between rat PAH and human PAH with the amount of divergence between

the TYH and PAH sequences. The rate of divergence between rat and human PAH in the regions covered by the incomplete rat PAH sequence (human PAH residues 208–452) was calculated to be approximately 7 PAMs/100 million years. This value is about average for the rate of mutation observed for other proteins among higher eukaryotes (Dayhoff, 1978). A similar calculation of the evolutionary distance between PAH and TYH was made by comparing the carboxyl-terminal regions of human PAH (residues 208–452) and rat PAH with rat TYH (residues 254–498). The TYH and PAH sequences differed by 51–55 PAMs. Assuming a constant rate of conservative evolution in which sequence divergence occurs by the gradual accumulation of point mutations, it is possible to estimate that TYH and PAH began to diverge as much as 700–800 million years ago.

Phenylalanine hydroxylase has been found in all vertebrates examined (Hseih & Berry, 1979) and has also been described in several species of bacteria (Letendre et al., 1984, 1975). The bacterial enzyme is a phenylalanine-specific, pterin-dependent hydroxylase that exhibits many of the characteristic properties of the enzyme of eukaryotes (Guroff et al., 1967) although its molecular weight is only 25 000–30 000 (Letendre et al., 1974, 1975). One species of bacteria, *Chromobacterium violaceum*, has two distinct pterin-dependent hydroxylases, one specific for phenylalanine and one specific for tryptophan (Letendre et al., 1974). Whether such hydroxylases share any homology with the differentiated hydroxylases of higher eukaryotes would be an interesting question.

It is interesting to speculate that the amino-terminal regions of TYH and PAH, which exhibit little or no homology to each other, may have arisen by a mechanism other than simple gene duplication and accumulation of point mutations. Such nonhomologous regions of proteins could arise by recruitment of unrelated exons carrying functional domains (Gilbert, 1978; Stone et al., 1985) or mutations in noncoding regions of the chromosomal gene that cause previously untranslated intronic sequences to be incorporated into the mature mRNA. Preliminary characterization of the genomic structure of PAH indicates that the gene is comprised of 14 exons extending over 120 kilobases (DiLella et al., unpublished observations). Of particular interest is the observation that an intron/exon boundary occurs between amino acids 117 and 118, immediately adjacent to the region homologous to TYH. This could represent the boundary of the homologous genetic structure containing the active core of the enzyme and lends support to the hypothesis of exon recruitment. It will be most interesting to compare the genomic structures of PAH and TYH for clues as to the evolutionary development of these proteins.

It is likely that TYH and PAH are only two members of a larger superfamily of enzymes. Tryptophan hydroxylase shares many biochemical properties with TYH and PAH (Kaufman & Fisher, 1974) and is likely to share considerable homology with TYH and PAH. It has also been suggested that dopamine β-hydroxylase and other enzymes in the catecholamine biosynthetic pathway share common determinants with TYH and PAH, even though they have different activities and mechanisms of action (Joh et al., 1983). Any homology among these proteins may be expected to provide important insight into their mechanisms of action and structure–function relationships and should provide further data on the evolution of these important enzyme systems.

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Resonance Raman Spectra of Extracellular Ligninase: Evidence for a Heme Active Site Similar to Those of Peroxidases

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ABSTRACT: The first resonance Raman spectra of the heme active site of the lignin-degrading enzyme ligninase are described. The strong correspondence between the spectra of ligninase and those of animal and plant peroxidases indicates that the local heme environment of ligninase greatly resembles those of peroxidases. By analogy with other heme-containing proteins it is likely that both the ferric and ferrous forms of ligninase are five-coordinate and high spin. The addition of cyanide to ferriligninase results in the formation of a low-spin six-coordinate heme active site. An iron-histidine stretching mode at ~244 cm^{-1} is suggested for ferroligninase in analogy with those of other peroxidases.

Lignin is a polymer of phenylpropanoid subunits that makes up 15-35% of lignocelluloses. The biodegradation of cellulose and hemicelluloses in woody plant tissues is hindered by the presence of lignin. The carbon cycle of this planet is most likely governed by lignin biodegradation (Millet et al., 1975; Kirk, 1983). In nature, this amorphous and complex polymer is decomposed mainly by higher basidiomycetous fungi that cause white rot of wood (Ander & Eriksson, 1978; Crawford, 1981). The major reaction involves the oxidative cleavage of the lignin propyl backbone between C_α and C_β. The lignin-degrading enzyme (ligninase) of the white-rot fungus *Phanerochaete chrysosporium* Burds. has been shown to catalyze this reaction in model compounds as well as spruce and birch lignins (Tien & Kirk, 1983). Ligninase also catalyzes the hydroxylations of benzylic methylene groups, the oxidation of phenols, and the oxidation of benzyl alcohols to their corresponding aldehydes or ketones. Very recently, this enzyme has been purified and partially characterized (Tien & Kirk, 1984; Gold et al., 1984). It has a molecular weight of ~42000 and requires H_2O_2 for its activity. The enzyme contains a single protoporphyrin IX, and EPR spectra (M. Tien and J. A. Fee, unpublished results) indicate that the iron is present as high-spin Fe^{3+}.

The mechanism by which this enzyme functions and the molecular origin of its ability to display optimal activity at extremely low pH (~2.5) are not yet completely understood. Recent studies have shown that aryl cation radicals are formed from the oxidation of substrates (Kersten et al., 1985; Hammel et al., 1985), and unpublished observations suggest that intermediates similar to those of compounds I and II of classical peroxidase reactions occur (M. Tien, C. Bull, and J. A. Fee, unpublished results).

Resonance Raman scattering studies of different hemoproteins have established that the vibrational spectra of the heme group provide a powerful means of characterizing the active sites of enzymes (Rousseau & Ondrias, 1983; Spiro, 1983). In this paper we describe the resonance Raman spectra of the heme group of ligninase. Our data show that the active site of this fungal protein is very similar to those of peroxidases and suggest that the high-spin Fe^{3+} of the native enzyme is five-coordinate. The reduced protein displays a mode (~250 cm^{-1}) that may be the analogue of the iron-proximal histidine stretching mode previously identified in horseradish peroxidase (Teraoka & Kitagawa, 1981).

Materials and Methods

Phanerochaete chrysosporium, strain BKM-1767 (ATTC 24725), was grown, and ligninase was purified to homogeneity according to Tien & Kirk (1984). All spectra were obtained from samples in ~0.2 M phosphate buffer at pH ~6.7 or in ~0.2 M tartrate buffer at pH 3-3.5. Reduced samples were

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