Tryptophan Fluorescence of Human Phenylalanine Hydroxylase Produced in *Escherichia coli*†

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ABSTRACT: Human phenylalanine hydroxylase (hPAH) contains three tryptophan residues (W120, W187, and W326). All three tryptophan residues were mutated to phenylalanine either as single mutants or in combination, and one tryptophan was also mutated to isoleucine. The mutant enzymes were expressed in *Escherichia coli* and purified as fusion proteins with maltose-binding protein and a linker region containing a recognition site for the serine protease factor Xa. After cleavage by factor Xa, all mutants were purified to homogeneity, and the kinetic and spectroscopic properties of the proteins were studied. All the proteins had high catalytic activities, but the affinity for phenylalanine was increased for the W120I and W120F mutants, and decreased for the W187F and W326F mutants. Using steady-state fluorescence spectroscopy, the contributions of the individual tryptophan residues to the total intrinsic fluorescence of the protein were estimated. On the basis of measurements of mutants containing only one tryptophan, it was calculated that W120, W187, and W326 account for approximately 61, 13, and 26% of the total tryptophan fluorescence of hPAH, respectively, while the positions of the emission maxima (335.5−336.5 nm) and the widths at half-height (55−60 nm) of the emission spectra of the individual tryptophans were rather similar. After incubation with phenylalanine, the quantum yield of wild-type hPAH increases by 15%, and the emission maximum is shifted from 336.5 to 347 nm. This effect is mainly due to changes in the W120 emission. On the basis of fluorescence quenching studies, this amino acid is the most surface-exposed of the tryptophan residues. The fluorescence of wild-type hPAH is partially (~50%) quenched by the addition of stoichiometric amounts of tetrahydrobiopterin. This is mainly (≥80%) due to quenching of the W120 fluorescence, while the W187 fluorescence is partially (22%) quenching by the addition of Fe(II). This characterization of the individual tryptophan residues and their specific interactions with ligands is valuable for future spectroscopic studies on the structure and function of hPAH.

Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH, EC 1.14.16.1)[1] is predominantly found in liver, and catalyzes the hydroxylation of L-phenylalanine to form tyrosine, using tetrahydrobiopterin (BH_4) and molecular oxygen as additional substrates [for a review, see Kaufman (1993)]. In humans, the absence or reduction of this enzyme activity leads to serious disease (phenylketonuria), and a number of naturally occurring mutations in the PAH gene have been detected in patients suffering from this disease (Eisensmith & Woo, 1992). Human PAH (hPAH) contains 3 tryptophan residues[2] (W120, W187, and W326) and 22 tyrosine residues (Kwok et al., 1985), which contribute to the absorbance and fluorescence spectra of the enzyme. The tryptophan residues present in hPAH are conserved in rat PAH (Dahl & Mercer, 1986), in *Drosophila* PAH (Morales et al., 1990), and in human tyrosine hydroxylase (TH) (Grima et al., 1987) as well as in rat TH (Grima et al., 1985). In human tryptophan hydroxylase, W120 and W187 are also conserved, but W326 is replaced by a phenylalanine (Boularand et al., 1990). Although the rat PAH enzyme has been crystallized (Celikel et al., 1991), the molecular structure is not known for any member of this protein family.

The intrinsic fluorescence of the rat liver PAH has previously been investigated in several laboratories. The fluorescence excitation maximum has been reported to be at about 280 nm, while the reported emission maxima range from 315 to 342 nm, using excitation at 280−295 nm (Marota & Shiman, 1984; Phillips et al., 1984; Haavik et al., 1986; Koizumi et al., 1988). Fluorescence lifetime studies on rat liver PAH have shown that the fluorescence emission can be resolved into two components with lifetimes of 1.7 and 4.1 ns, respectively (Koizumi et al., 1988). Phenylalanine binds with positive cooperativity to PAH, and the binding of this amino acid (as well as structurally related

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[1] Abbreviations: PAH, phenylalanine hydroxylase; hPAH, human PAH; MBP, maltose-binding protein; IPTG, isopropylthio-β-D-galactopyranoside; one-letter abbreviations W, F, and I, are for tryptophan, phenylalanine, and isoleucine, respectively; W120F, W187F, and W326F represent mutant proteins in which tryptophans at a particular position have been substituted by another amino acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

[2] On the basis of amino acid analysis, rat phenylalanine hydroxylase was previously assumed to contain three (Døskeland et al., 1982) or four (Shiman et al., 1980; Phillips et al., 1984) tryptophan residues per subunit. Subsequent studies using cDNA sequencing have shown that both the rat and human enzymes contain three tryptophans per subunit (Kwok et al., 1985).

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Table 1: Oligonucleotides Used for PCR-Based Mutagenesis[a]

primer	sense	cDNA position	sequence (5′−3′)
A120F	forward	571−588	ACAGTGCCC**T**CTTCCCA
A120I	forward	571−588	ACAGTGCCC**AT**CTTCCCA
A187F	forward	766−785	GAAGAAAAGAAAACATT**C**GG
A326F	forward	1186−1203	GCCACAATTTACT**T**CTTT
C_1	forward	MBP	GGTCGTCAGACTGTCGATGA
C_2	forward	932−949	GCACTGGTTTCCGCCTCC
D	inverse	none	CTGCCCATTCCTCATGTAGA
B_{40I}	inverse	1078−1098	CTGCCCATTCCTCATGTAGA-
B_{40II}	inverse	1523−1543	CTGCCCATTCCTCATGTAGA-TTTCACTGTTAATGGAATCA

[a] The underlined sequences correspond to PAH cDNA. Mutant nucleotides are shown in boldface type. Primer D is identical to the first 20 nucleotides of primers B_{40I} and B_{40II} (Dworniczak et al., 1991).

compounds and detergents) leads to changes in the enzyme conformation, as revealed by an altered oligomerization state, altered affinity for protein kinases, and spectroscopic changes (Døskeland et al., 1982, 1984; Phillips et al., 1984; Parniak, 1990). The spectroscopic changes include altered electron paramagnetic resonance (EPR) spectra of the enzyme-bound iron (Wallick et al., 1984) and perturbed circular dichroism and far-UV absorbance spectra (Phillips et al., 1984), in addition to the changes in tryptophan fluorescence. The fluorescence changes include an 8−20 nm red-shift of the emission maximum and an increased quantum yield by incubation with phenylalanine, while the addition of BH_4 partially quenches the fluorescence, without any shift of the fluorescence maximum (Phillips et al., 1984; Haavik et al., 1986; Koizumi et al., 1988, Shiman et al., 1990). Important aims of the present study were to compare the recombinant human enzyme with the rat liver enzyme, to examine contributions of the different tryptophan residue(s) to the fluorescence spectra, and to identify the residue(s) responsible for the spectroscopic changes induced by ligand binding. This was achieved by generating seven W→F and one W→I substitution mutants and comparing the spectroscopic properties of the mutant proteins with the wild-type hPAH. Since some of the mutant proteins had altered regulatory properties, it was also necessary to characterize the kinetic properties of the mutants, in order to interpret the effects of tryptophan replacements.

MATERIALS AND METHODS

Materials. Guanidine hydrochloride, urea, potassium iodide, and cesium chloride were obtained from E. Merck. Factor Xa and enteropeptidase (enterokinase) were from Boehringer Mannheim and Biozyme Laboratories Ltd., respectively. (6*R*)-Tetrahydrobiopterin (BH_4) and 6-methyltetrahydropterin were from Schircks Laboratories. L-Phenylalanine, L-tryptophan, acrylamide, and isopropyl thio-β-D-galactopyranoside (IPTG) were from Sigma. These as well as the other reagents used in this study were of analytical grade.

Site-Directed Mutagenesis of PAH. To mutate the three different tryptophan residues in the hPAH enzyme to either phenylalanine or isoleucine, the PCR-based procedure described by Nelson and Long (1989) was applied. The primers used in the mutagenesis are listed in Table 1. The target sequences for mutagenesis were the *Xba*I/*Xho*I, *Xho*I/*Bam*HI, and *Bam*HI/*Afl*II fragments of the hPAH cDNA for the W120, W187, and W326 residues, respectively. Positive clones were identified by the loss of a *Bst*NI restriction site (W120F/W120I), the creation of an *Xmn*I site (W187F), or the loss of a *Bsr*I site (W326F). The authenticity of the mutagenesis was verified by DNA sequencing.

Expression and Purification of Recombinant Human PAH. The wild-type hPAH, as well as all the mutants described here, were expressed in high yield in *E. coli* as fusion proteins with maltose-binding protein (MBP)[3] (Martinez et al., 1995) from the pMALPAH vector (Knappskog et al., 1995), and were purified to homogeneity using a single affinity chromatographic step on an amylose resin (New England Biolabs) and subsequent size-exclusion chromatography on a Sephacryl S-300 column (Pharmacia). The affinity chromatography was performed as described by the manufacturer of the affinity resin, and the details of the purification of the wild-type enzyme have recently been described by Martinez et al. (1995) The fusion proteins were cleaved by incubation with factor Xa for 12−16 h, or with enteropeptidase (enterokinase) for 24 h at 0 °C, and hPAH was separated by chromatography on a Superdex-75 (1 × 16 cm) gel filtration column (Pharmacia) from MBP and the "restriction proteases". The ratio of fusion protein to protease was 500:1 (mg/mg) for factor Xa and 50:1 for enteropeptidase. Both wild-type hPAH and the mutants eluted from the gel filtration column as mixtures of several oligomeric species (predominantly dimers and tetramers). Since these species have been shown to be interconvertible in the case of rat and bovine PAH (Døskeland et al., 1982; Parniak, 1990), it was decided to include both species in the subsequent purification. The enzymes were concentrated by ultrafiltration on Centricon-30 units (Amicon), and the purity was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and by determination of the ratio of absorbance at 278 and 280 nm (see below).

Fluorescence Measurements. Unless specifically stated, all experiments with hPAH were performed in 20 mM Na-Hepes buffer, pH 7.0, containing 150−200 mM NaCl, with the protein concentration adjusted to an optical density of approximately 0.02 at the excitating wavelength. All experiments were performed at 25 °C, using a Perkin-Elmer LS-50 luminescence spectrometer, with a constant-temperature cell holder and 1 cm path-length quartz cells with maximal stirring. The quantum yield of protein fluorescence was determined using tryptophan in water as a secondary standard ($Q = 0.14$; Eisinger, 1969). All spectra were corrected for blank emission and the inner filter effect (where appropriate).

Spectrophotometry. Ultraviolet spectra were obtained using Cary 219 and Hewlett Packard 8452A spectrophotometers.

Determination of Protein Concentration. Since the mutation of tryptophans leads to changes in the UV absorbance spectra of hPAH, an alternative method of protein determination was needed. The protein determination method described by Bradford (1976) and later refined by other investigators was selected, due to its simplicity and sensitivity, and since the color response is only slightly dependent on the content of aromatic amino acids (Compton & Jones, 1985). The wild-type hPAH ($A_{280}^{1\%} = 10.0$ for the rat

[3] The intact fusion protein (with maltose-binding protein) is referred to as "fusion protein", while the purified PAH (without the fusion partner) is termed "pure PAH".

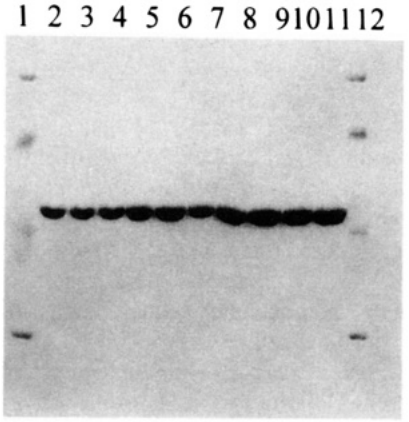

FIGURE 1: SDS−polyacrylamide gel electrophoresis of the wild-type and mutant hPAHs. Lane 2, purified wild-type PAH (factor Xa); lane 3, wild-type PAH (enteropeptidase); lane 4, W120I PAH; lane 5, W120F PAH; lane 6, W187F PAH; lane 7, W326F PAH; lane 8, W120F/W187F PAH; lane 9, W120F/W326F PAH; lane 10, W187F/W326F PAH; lane 11, W120F/W187F/W326F PAH. Lanes 1 and 12 contains standard proteins with molecular masses of 97.7, 66.2, 45, and 31 kDa, respectively. Electrophoresis was performed under denaturing conditions on gels containing 10% polyacrylamide. The gel was stained by Coomassie brilliant blue.

enzyme; Shiman et al., 1979) was used as a standard, and the estimated extinction coefficients of the mutants were corrected to account for these predicted differences (0.2−0.7%) (Compton & Jones, 1985).

Quenching Studies. For these studies, the absorbance at the excitation wavelength was less than 0.05. During addition of ionic quenchers (KI and CsCl), the ionic strength, pH, and temperature were kept constant by dissolving the enzyme in an equal concentration of buffered sodium chloride (Arrio-Dupont, 1978). The quenching data were analyzed according to the Stern−Volmer equation and the modified Stern−Volmer equation (Lehrer, 1971).

Measurement of PAH Activity. The activity of PAH was assayed at 25 °C as previously described (Døskeland et al., 1982). In brief, the assay mixture contained 1 mM phenylalanine, 100 μM Fe(II), 0.25 mM tetrahydrobiopterin (BH_4), and 100 mM Na-Hepes, pH 7.0. The enzyme was preincubated for 5 min in the presence of phenylalanine, and the assay was started by the addition of tetrahydropterin. The amount of L-tyrosine formed was determined using HPLC with fluorometric detection (Dϕskeland et al., 1984). The kinetic data were analyzed using nonlinear regression (the program ENZ-FIT[4]).

RESULTS

Purification of the PAH Mutants. The wild-type hPAH and the mutant enzyme forms described in this study were expressed in high levels in *E. coli* as fusion proteins with MBP and a linker region, containing a recognition site for the serine protease factor Xa. In addition, for the wild-type hPAH, a similar construct was prepared with the linker region containing a recognition site for enteropeptidase (enterokinase). All the enzymes were purified to homogeneity, as judged by SDS−PAGE, using a single affinity chromatographic step on an amylose resin and subsequent size-exclusion chromatography (Figure 1). The purification and chromatographic properties of the wild-type fusion protein have recently been described (Martinez et al., 1995). The mutants containing the W187F mutation eluted as two equal peaks from the amylose resin column, while wild-type hPAH and all the other mutants were eluted in a single peak. Furthermore, a large fraction (50−80%) of the fusion proteins containing the W187F mutation in PAH formed high molecular weight aggregates of inactive protein (M_r >1 000 000, as judged by Sephacryl S-300 chromatography), while only about 20−30% of the protein formed tetramers, with high catalytic activity. The fraction of apparently dimeric protein was also increased for these mutants. In comparison, only about 15−25% of the wild-type protein, and the other single mutants studied, formed such high molecular weight aggregates of inactive protein (Table 2). This suggests that the W187F mutation has an effect on the oligomeric structure of the fusion protein. Except for the W120F mutant, the yields of all the mutant fusion proteins (Table 2) and purified proteins (data not shown) were lower than that of the wild-type protein, possibly due to altered physicochemical properties of the mutant proteins.

Table 2: Yield and Oligomeric State of the Different hPAH Fusion Proteins during Purification by Sephacryl S-300 Column Chromatography

enzyme	average yield of tetrameric hPAH (mg)[a]	fraction of hPAH eluted as inactive aggregates (% of total)
wild-type (enteropeptidase)	40.0	18
wild-type (factor Xa)	35.0	24
W120I	9.4	20
W120F	52.0	20
W187F	15.0	43
W326F	20.0	25
W120F/W187F	0.8	84
W120F/W326F	8.5	45
W187F/W326F	1.8	80
W120F/W187F/W326F	0.8	80

[a] The yield of tetrameric fusion proteins per liter of bacterial culture is indicated (average of 2−6 experiments). This fraction of the protein was used in the subsequent purification (see Materials and Methods).

Kinetic Properties of the hPAH Fusion Proteins. The specific activity of the wild-type hPAH and the mutants was measured before and after cleavage by factor Xa or enteropeptidase, since some of the mutants were less stable after cleavage. The activity of the wild-type enzyme is increased by 10−30% when Fe(II) is added to the assay mixture (Martinez et al., 1995). For this reason, 100 μM Fe(II) ammonium sulfate was included in the assay mixture of all the enzyme forms. The kinetic properties of representative preparations of the fusion proteins are shown in Table 3. The specific activities of the enzymes showed up to 50% batch-to-batch variations, and decreased rapidly on freezing and thawing. This may also be a partial explanation for the variable yield of enzyme during purification (Table 2). In contrast, the measured affinities for phenylalanine and BH_4 were reproducible for the different enzyme batches. As shown in Table 3, the affinity for BH_4 was little influenced by the W→F and W→I mutations, while there was a 6-fold range in the affinity for phenylalanine among the different mutants. Also, under the selected assay conditions, there was no substrate inhibition by BH_4 at concentrations up to 10 times the K_m values, while phenylalanine was inhibitory at high concentrations.

[4] The ENZ-FIT program was developed by F. W. Perella, Medical Products Department, E. I. du Pont de Nemours and Co.

Table 3: Kinetic Properties of the hPAH Fusion Proteins[a]

enzyme	V_{max} (nmol mg^{-1} min^{-1})	$K_m(BH_4)$ (μM)	$S_{0.5}$(Phe) (μM)	Hill constant (Phe)
wild-type (enteropeptidase)	461 ± 8	15.9 ± 1.0	120 ± 18	2.5 ± 0.6
wild-type (factor Xa)	472 ± 22	23.9 ± 3.6	121 ± 7	2.0 ± 0.2
W120I	999 ± 170	24.1 ± 4.0	nd[b]	nd
W120F	160 ± 8	15.4 ± 3.0	65 ± 7	1.3 ± 0.1
W187F	712 ± 68	21.8 ± 5.8	260 ± 5	2.4 ± 0.1
W326F	355 ± 14	17.7 ± 1.8	367 ± 5	2.3 ± 0.1
W120F/W187F	110 ± 5	14.7 ± 2.1	224 ± 25	1.3 ± 0.2
W120F/W326F	372 ± 17	12.6 ± 2.0	101 ± 2	1.7 ± 0.1
W187F/W326F	116 ± 7	24.9 ± 3.7	326 ± 20	2.2 ± 0.3
W120F/W187F/W326F	153 ± 7	26.1 ± 3.4	202 ± 23	1.3 ± 0.1

[a] Standard assay conditions (see Materials and Methods) and variable concentrations of phenylalanine (0−4 mM) and BH_4 (0−0.15 mM). The numbers given represent the mean values ± SEM using nonlinear regression. [b] nd, not determined.

Kinetic Properties of the Purified hPAH Mutants. After cleavage by the "restriction proteases" and purification of the hPAH mutants, the catalytic activities of the enzymes were generally well conserved. However, all the hPAH mutants containing the W120F mutation, either as a single mutation or in combination with others, gradually precipitated, suggesting that W120 is critical for the conservation of the correct conformation/oligomeric state of the cleaved protein, and that even substitution with another large neutral amino acid like phenylalanine perturbs the tertiary structure of hPAH. In order to determine whether this phenomenon was specific for the W→F substitution, the W120I mutant was also prepared. However, this mutant precipitated even faster than the W120F mutants, precluding any detailed spectroscopic characterization of this protein. For both W120F and W120I, analysis of the precipitated protein by SDS−PAGE showed that it consisted of >90% pure hPAH, while the MBP fusion partner stayed in solution. For the W120F mutants, the precipitation occurred relatively slowly (apparent $t_{1/2}$ of 4−6 h at 0−5 °C), permitting the isolation of a soluble fraction of the enzyme. However, even this fraction of the W120F/I mutants precipitated if they were kept for several hours at 0−4 °C.

It has recently been shown that factor Xa cleaves the wild-type fusion protein at two sites, i.e., both between Arg in the linker region and Ser-2 in hPAH and between Arg-13 and Lys-14 in hPAH (Martinez et al., 1995), while enterokinase only cleaves the protein at the expected site between the linker and Ser-2 in hPAH. Attempts to increase the specificity of the factor Xa cleavage by reversible acylation of the fusion protein (Wearne, 1990) were unsuccessful. In order to obtain some 100% intact hPAH, the wild-type enzyme was also prepared by enteropeptidase cleavage. However, since the rate of this cleavage was at least 1 order of magnitude slower than the cleavage by factor Xa, the factor Xa cleaved enzyme was selected for a more detailed analysis. All the enzymes were purified to homogeneity as judged from SDS−PAGE (Figure 1), and from the ultraviolet spectra of the proteins (see Materials and Methods). Since MBP (M_r = 42 479) contains eight tryptophan residues, and the PAH mutants (subunit M_r = 51 728 for the wild-type hPAH) contained from zero to three tryptophans, it was important to remove any traces of the fusion partner during the final purification.

All the fusion proteins, as well as the purified enzyme forms, displayed sigmoidal activity curves as a function of added phenylalanine, and were inhibited at high concentrations of this substrate. The W120F and the W120I mutants had a 3−6-fold increased affinity ($S_{0.5}$) for phenylalanine,

Table 4: Kinetic Properties of the Purified hPAH Proteins[a]

enzyme	V_{max} (nmol mg^{-1} min^{-1})	$S_{0.5}$(Phe) (μM)	Hill constant (Phe)
wild-type (enteropeptidase)	702 ± 47	121 ± 14	2.1 ± 0.7
wild-type (factor Xa)	1000 ± 20	110 ± 13	2.4 ± 0.1
W120I	83 ± 3	21 ± 2	1.4 ± 0.2
W120F	470 ± 28	47 ± 6	1.7 ± 0.3
W187F	770 ± 18	189 ± 6	2.8 ± 0.1
W326F	584 ± 12	432 ± 11	2.3 ± 0.1
W120F/W187F	286 ± 25	104 ± 17	2.1 ± 0.5
W120F/W326F	794 ± 18	155 ± 10	2.4 ± 0.2
W187F/W326F	550 ± 3	471 ± 4	2.6 ± 0.1
W120F/W187F/W326F	483 ± 23	258 ± 25	1.5 ± 0.1

[a] The enzymes were assayed as described in Table 3.

while the W187F and W326F mutants showed a decrease in the affinity for this substrate (Table 4). The extent of the positive cooperativity, as expressed by the Hill coefficient for the phenylalanine activation (n_H), varied inversely with the phenylalanine affinity ($S_{0.5}$); (Table 3 and 4). However, as these correlations were far from perfect, one may speculate that the W→F mutations not only change the equilibrium between unactivated and activated hPAH but may also influence the binding affinity for phenylalanine by other mechanisms. Interestingly, the hPAH mutants with multiple W→F mutations had phenylalanine affinities and Hill coefficients which appeared to be intermediate between those of the individual mutants. The wild-type PAH and all the mutants (fusion proteins and purified hPAHs) showed hyperbolic kinetics and rather similar K_m values with the substrate BH_4 (Table 3 and data not shown).

Ultraviolet Absorbance Spectra. As expected, the UV absorbance spectra were altered for the mutants where one, two, or three tryptophans were substituted with phenylalanines, with a 44% reduction in the absorbance at 280 nm for the "triple mutant" compared to the wild-type. Since hPAH contains 3 tryptophan and 22 tyrosine residues per subunit, while the expression partner (MBP) contains 8 tryptophans and 15 tyrosines, the UV spectra, and more specifically the ratio of absorbance at 278/280 nm, were different for the two proteins (absorbance ratio 1.007 and 0.990, respectively). This ratio was even higher for the W→F mutated hPAHs (A_{278}/A_{280} = 1.012, 1.025, and 1.032 for the single, double, and triple mutants, respectively). This property was useful not only to determine the purity of the enzyme preparations but also as a fast method to determine which fractions contained the purified PAH during separation of the two proteins by gel filtration chromatography.

Fluorescence Spectra. Both wild-type hPAH and the single and double tryptophan mutants described here had

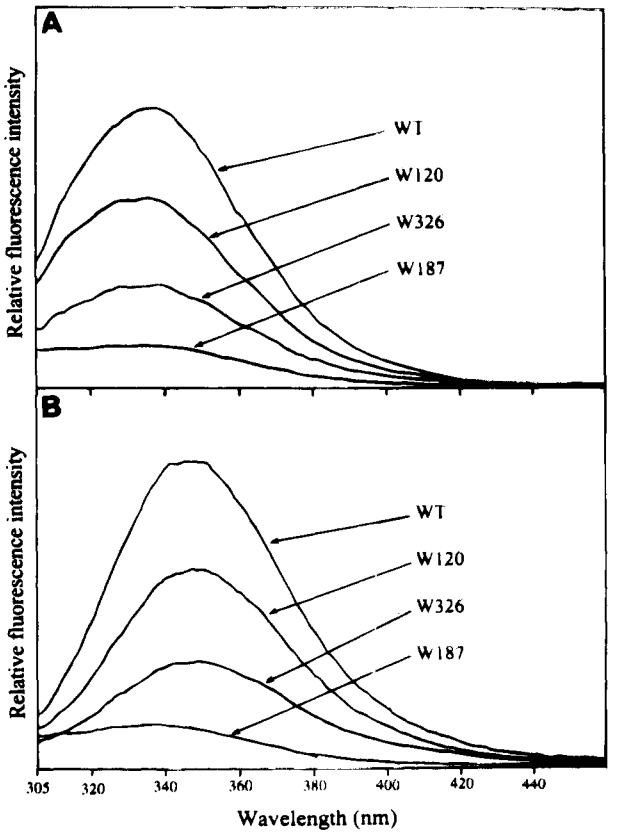

FIGURE 2: Fluorescence emission spectra of wild-type and double mutants containing only W120, W187, or W326 in the absence (A) and in the presence of 1 mM phenylalanine (B). Approximately 40 μg/mL enzyme in 20 mM Na-Hepes, pH 7.0, containing 200 mM NaCl was used. All spectra were obtained using an excitation wavelength of 295 nm and excitation and emission slits of 5 and 2.5 nm, respectively.

Table 5: Quantum Yields and Fluorescence Emission Maxima of Native and Phenylalanine Activated Enzymes

	Q^a		emission maximum (nm)	
enzyme	no additions	+Phe (1 mM)	no additions	+Phe (1 mM)
wild-type (enteropeptidase)	0.086	0.091	341.8	346.7
wild-type (factor Xa)	0.088	0.101	336.5	347.0
W120F	0.025	0.028	338.0	340.5
W187F	0.061	0.064	338.0	346.7
W326F	0.110	0.123	335.0	348.0
W120F/W187F	0.043	0.046	336.5	348.0
W120F/W326F	0.021	0.020	336.5	339.0
W187F/W326F	0.100	0.111	335.5	348.5

[a] The quantum yield of the different mutants was calculated based on the fluorescence emission from 305 to 500 nm, using excitation at 295 nm, and free tryptophan in water (Q = 0.14; Eisinger, 1969) as the secondary standard. The results shown are the average of three different experiments. All the mutant proteins were obtained by factor Xa cleavage of fusion proteins.

fluorescence excitation and emission spectra typical of partially solvent-exposed tryptophan residues ($\lambda_{max\ excit}$ = 281 nm, $\lambda_{max\ em}$ = 335−338 nm, widths at half-height of 55−60 nm) (Figure 2 and Table 5; Burstein et al., 1973). When the pH was raised from pH 7.0 to pH ≥9.0, the emission maximum of the wild-type factor Xa cleaved hPAH shifted from 336.5 to 347 nm, and the fluorescence intensity increased to the level observed at saturating levels of phenylalanine. The apparent pK_a for this transition was about 8.0. After incubation at alkaline pH, no additional effects of added phenylalanine on the emission spectra were observed. A similar shift of the emission maximum was observed after repeated freezing and thawing of the protein, and this effect may also explain the differences in emission maxima observed for the fusion proteins cleaved by factor Xa and enteropeptidase (Table 5), since the enteropeptidase-cleaved enzyme had been subjected to protease treatment several hours longer than the factor Xa cleaved enzyme. In the presence of 7 M urea or 6 M guanidine hydrochloride, the emission maxima of all the mutants shifted to about 354 nm, as expected for completely solvent-exposed tryptophan residues (Burstein et al., 1973).

Measurement of the quantum yields of the wild-type and mutant hPAHs showed that W120 accounts for about two-thirds of the total tryptophan fluorescence of hPAH and that W187 is the least fluorescent of the three tryptophans (Table 5). However, the quantum yields of the multiple tryptophan proteins could not be accurately predicted from the fluorescence of the single tryptophan mutants. This may be due to energy transfer between individual tryptophans in the protein, or that the W→F mutations also affect the physicochemical properties, including the spectroscopic features, of the remaining tryptophans in the protein. Since the majority of the wild-type and mutant proteins were isolated in the tetrameric state, energy transfer may occur within the individual subunits of the enzyme, as well as between the subunits of the oligomer. However, since the emission spectra of the individual tryptophans were rather similar, and the kinetic properties of the W→F and W→I mutants were somewhat different from those of the wild-type hPAH (Tables 2−4), the latter explanation is the most likely.

Effect of Phenylalanine on the Fluorescence. In the presence of phenylalanine, the emission maximum of the wild-type enzyme was red-shifted by approximately 10 nm (from 336.5 to 347 nm) (Figure 2) and the fluorescence intensity (quantum yield) increased by about 15% (Table 5). The concentration dependency and the time course of these changes paralleled the increases in specific activity observed by incubation with phenylalanine (data not shown), supporting the view that the putative conformational changes observed by fluorescence spectroscopy are also essential for enzyme activation (Phillips et al., 1984). The effect of phenylalanine addition was most pronounced on preparations of high specific activity, indicating the need for a native protein. Similar effects of added phenylalanine were also observed on the fluorescence of the fusion protein, but due to the high tryptophan content of the MBP, the magnitude of the changes was only about 20% of that observed for the isolated hPAH (data not shown). This spectroscopic change, as well as the enzyme cooperativity observed for the fusion proteins (Table 3), indicates that the fusion partner does not hinder the conformational changes of hPAH induced by substrate binding. Similar experiments were also performed with all the mutants described in this study. Correcting for the differences in quantum yields of the different tryptophan residues, the contributions from W120 and W326 to the total increase in fluorescence induced by phenylalanine were calculated to be about 80 and 20%, respectively (Table 5). The observation that all three tryptophans, although widely separated in the protein sequence, are affected by the phenylalanine binding is compatible with a major conformational change in the protein after ligand binding.

Effect of Added Metal Ions. The fluorescence intensity of the wild-type hPAH was moderately quenched (9% decrease in fluorescence intensity), without any shift of emission maximum, on addition of stoichiometric amounts of Fe(II). The quenching was partially reversed by the subsequent addition of a 1000-fold excess of EDTA, which indicates that it was due to reversible binding to the active site of the enzyme (Haavik et al., 1992). The selective stimulation of enzyme activity in the presence of micromolar concentrations of Fe(II) also supports such a conclusion. Similar experiments were also performed on the mutant proteins. Of the hPAH mutants containing only a single tryptophan residue, the fluorescence of the W120F/W326F mutant (containing W187) was rapidly quenched to a stable level (22%) by the addition of Fe(II). The quenching was slowly reversed by the addition of EDTA, and an apparent end point was observed at 3−4 equiv of added Fe/enzyme subunit. This indicates either that the quenching cannot be explained by the binding to a single metal-binding site, as reported for human TH (Haavik et al., 1992), or that the affinity is too low to give a defined end point at the concentrations used. Interestingly, in the primary structure, W187 is situated close to the histidine residues postulated to be involved in the binding of metals (Gibbs et al., 1993; Andersson et al., 1988). The fluorescence of the W120 and W326 single tryptophan mutants was minimally affected by the addition of up to 10 equiv of Fe(II). The wild-type (factor Xa cleaved) enzyme used in these experiments contained ≥0.35 equiv of iron per subunit (A. Martinez, personal communication), and this may explain why the relative fluorescence quenching was much less than that observed for metal-free TH (Haavik et al., 1992).

Effect of Added Tetrahydrobiopterin on the Fluorescence Intensity. When BH_4 was added to wild-type hPAH, a concentration-dependent decrease of the fluorescence was observed. An apparent end point was reached at a BH_4/PAH ratio of about 1.0, which is in agreement with the fluorescence quenching reported for the rat liver enzyme (Phillips et al., 1984; Haavik et al., 1986; Shiman et al., 1994). When this experiment was repeated with the mutants containing single tryptophan residues, the fluorescence quenching was most pronounced for the mutant containing only W120. As for the wild-type hPAH, the fluorescence of this mutant was quenched by about 50%, in the presence of equimolar concentrations of BH_4, while the fluorescence of the other single tryptophan proteins was quenched by 10−20% at this concentration BH_4 (Figure 3). Thus, the fluorescence quenching of W120 probably accounts for more than 80% of the quenching of the wild-type hPAH by BH_4.

Fluorescence Quenching by Ionic Quenchers. The tryptophan fluorescence of proteins can be quenched by many different substances. In particular, iodide ions can quench the fluorescence of surface-exposed tryptophan residues, but cannot penetrate into the hydrophobic core of proteins. Iodide quenching of wild-type and mutant proteins was performed as described under Materials and Methods. Except for the situation where the enzyme was in the completely denatured state (with 7 M urea, results not shown), all the quenching curves were found to be nonlinear, as expected for a protein with partial quenching of a heterogeneous population of tryptophan residues. The fraction accessible for quenching (f_a) in the unactivated wild-type PAH was about 0.53, and this fraction increased to 0.59 in the presence of 1 mM phenylalanine (Table 6). Likewise, the Stern−Volmer quenching constant with KI of the wild-type hPAH increased 2.6-fold (from 1.6 M^{-1} to 4.2 M^{-1}) in the presence of 1 mM phenylalanine. A similar effect was seen when CsCl was used as the quencher. In this instance, the Stern−Volmer constant increased from 1.9 M^{-1} (f_a = 0.13) without phenylalanine to 3.8 M^{-1} (f_a = 0.18) in the presence of 1 mM phenylalanine. This indicates that phenylalanine activation leads to an increased exposure of tryptophans both to the solvent (Figure 4) and to ionic quenchers (Table 6). A comparison of the emission spectra before and after quenching with KI showed that the tryptophans which were exposed by treatment with phenylalanine were selectively quenched by KI (data not shown).

The fluorescence of the W187F/W326F double mutant (where W120 is intact) was efficiently quenched by KI (f_a = 0.91) (Table 6). In contrast, a small fraction of the fluorescence of the double mutants containing only W187 or W326 was quenched by KI (f_a = 0.07 and 0.23, respectively). Since these mutants were all single tryptophan proteins, part of the fluorescence quenching may indeed be due to the presence of small amounts of denatured protein in the enzyme preparations, containing solvent-exposed tryptophans. Together, these data suggest that W120 is the

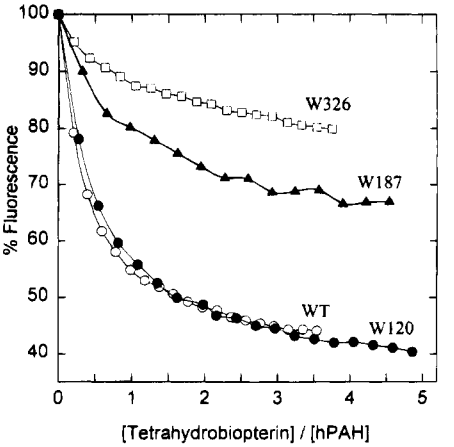

FIGURE 3: Quenching of the fluorescence of wild-type and mutants containing only one tryptophan (W120, W187, or W326) by added tetrahydrobiopterin. Approximately 40 μg/mL enzyme was incubated in 20 mM Na-Hepes, pH 7.0, 0.5 mM DTT, and 0.1 mM Fe(II)SO_4 for 5 min at 25 °C before titration with tetrahydrobiopterin. The excitation and emission wavelengths were 295 and 340 nm, respectively, and the slits were 5 and 15 nm.

Table 6: KI Quenching Constants (K_{sv}) and Fraction of Fluorescence Quenched of the Wild-Type and Single Tryptophan hPAHs[a]

	K_{sv} (M^{-1})		fraction accessible for quenching (f_a)	
enzyme[b]	no additions	+Phe (1 mM)	no additions	+Phe (1 mM)
wild-type hPAH	1.6	4.2	0.53	0.59
W120F/W187F	5.5	6.5	0.23	0.32
W120F/W326F	5.9	10.9	0.07	0.08
W187F/W326F	1.7	5.7	0.91	0.70

[a] The quenching constants and fractions accessible for quenching were determined as described by Lehrer (1971), using the modified Stern−Volmer equation and concentrations of KI ranging from 0.05 to 0.6 M. Due to the low quenching of W120F/W187F and W120F/W326F by iodide, the precision of the quenching parameters for these proteins is lower than for the two other proteins. [b] All proteins were obtained by factor Xa cleavage of fusion proteins.

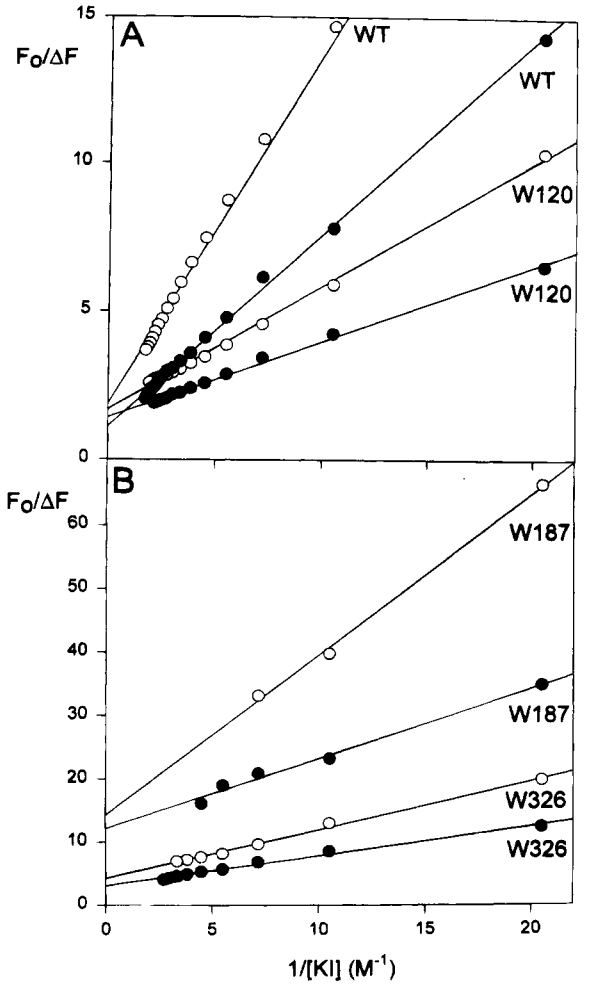

FIGURE 4: Modified Stern–Volmer plots for the iodide quenching of the wild-type and the double mutant containing only W120 (A) and W187 and W326 (B) in the absence (○) and in the presence of 1 mM phenylalanine (●). The conditions were as described under Materials and Methods and the excitation and emission wavelengths as described in the legend to Figure 3.

most fluorescent and solvent-exposed of the three tryptophan residues in hPAH. After addition of 1 mM phenylalanine, there was a 3.4-fold increase in the KI quenching constants for W120, while the quenching of W187 and W326 was moderately increased. Correcting for the low quantum yields of these tryptophans compared to W120 (Table 5), it is evident that W120 is almost exclusively responsible for the increased KI and CsCl quenching of wild-type hPAH after addition of phenylalanine.

Acrylamide Fluorescence Quenching. Acrylamide is a neutral polar quencher which can gain access to deeply buried tryptophan residues, ideally without being influenced by local charges. Thus, when acrylamide was used to quench the intrinsic fluorescence of the various hPAH mutants, the Stern–Volmer plots either were linear or showed an upward deviation (Figure 5). The wild-type hPAH and the mutant containing only W120 had the highest quenching constants, and the Stern–Volmer plots of these two enzyme forms were virtually identical (Figure 5). This is consistent with W120 being the main fluorophor in the wild-type hPAH, as described above. For these enzyme forms, the quenching constants were significantly increased in the presence of 1 mM phenylalanine (Figure 5 and Table 7), while the fluorescence quenching of the mutants containing only W187 or W326 was not as sensitive to the presence of the substrate. The upward deviation of the Stern–Volmer plots could be due to a static quenching component, or to a conformational change of the protein occurring at high concentrations of acrylamide. In the absence of complete data on fluorescence lifetimes, it is difficult to estimate the contribution of static quenching to the total acrylamide quenching (Eftink et al., 1981). However, since all the quenching curves were almost linear in the presence of 1 mM phenylalanine, it is most likely that high concentrations of acrylamide induce a conformational change similar to that induced by substrate binding, possibly by interaction with the phenylalanine binding site(s). The binding of acrylamide to the amide/carboxylic acid binding sites of proteins has previously been reported for liver alcohol dehydrogenase (Eftink et al., 1981), and it is known that rat PAH can be activated by many different substances in addition to L-phenylalanine, including the other natural hydrophobic L-amino acids (Shiman et al., 1994). The effect of acrylamide on hPAH activation was confirmed by examining the effect of acrylamide on the phenylalanine activation curves. In the presence of 1 M acrylamide, hPAH is showing "pure" hyperbolic kinetics (n_H = 1.0, K_m = 55 μM), with increased activity at low concentrations of phenylalanine and inhibition at high concentrations (Figure 6), as expected from a heterotropic interaction with the phenylalanine binding/activation site. Since even low concentrations of acrylamide seem to induce this conformational change, the Stern–Volmer constants were extrapolated from the initial slopes of the quenching curves (Figure 5, Table 7).

Table 7: Acrylamide Quenching Constants (K_{sv}) of the Wild-Type hPAH and Single Tryptophan Mutants[a]

enzyme[c]	K_{sv} (M^{-1}) no additions	K_{sv} (M^{-1}) +Phe (1 mM)
wild-type hPAH	2.2[b]	5.5
W120F/W187F	1.6[b]	1.8
W120F/W326F	3.5[b]	3.1
W187F/W326F	2.0[b]	5.5

[a] The quenching constants were calculated using the Stern–Volmer equation and concentrations of acrylamide ranging from 0.05 to 0.55 M. [b] Since the acrylamide quenching curves were showing an upward deviation, and this was partly due to a conformational change mimicking the effect of phenylalanine, the constants for the protein without phenylalanine preincubation were extrapolated from the lowest concentrations of acrylamide. [c] All proteins were obtained by factor Xa cleavage of the respective fusion proteins.

DISCUSSION

Here we have shown that the MBP fusion proteins of both wild-type hPAH, several W→F mutants, and one W→I mutant can be expressed at high levels in *E. coli* and purified as active proteins with apparently intact regulatory properties. While previous attempts to obtain native wild-type and mutant hPAH enzyme forms in the pET expression system have been relatively unsuccessful, due to the rapid degradation of hPAH in the bacteria (Knappskog et al., 1993), such problems have not been observed using the pMAL system (Martinez et al., 1995). It is also in contrast to the recent expression of rat PAH in *E. coli*, which is reported to give an inactive protein (Gibbs et al., 1993). The expression and purification of human tryptophan hydroxylase as a fusion protein with MBP have also been reported (Yang &

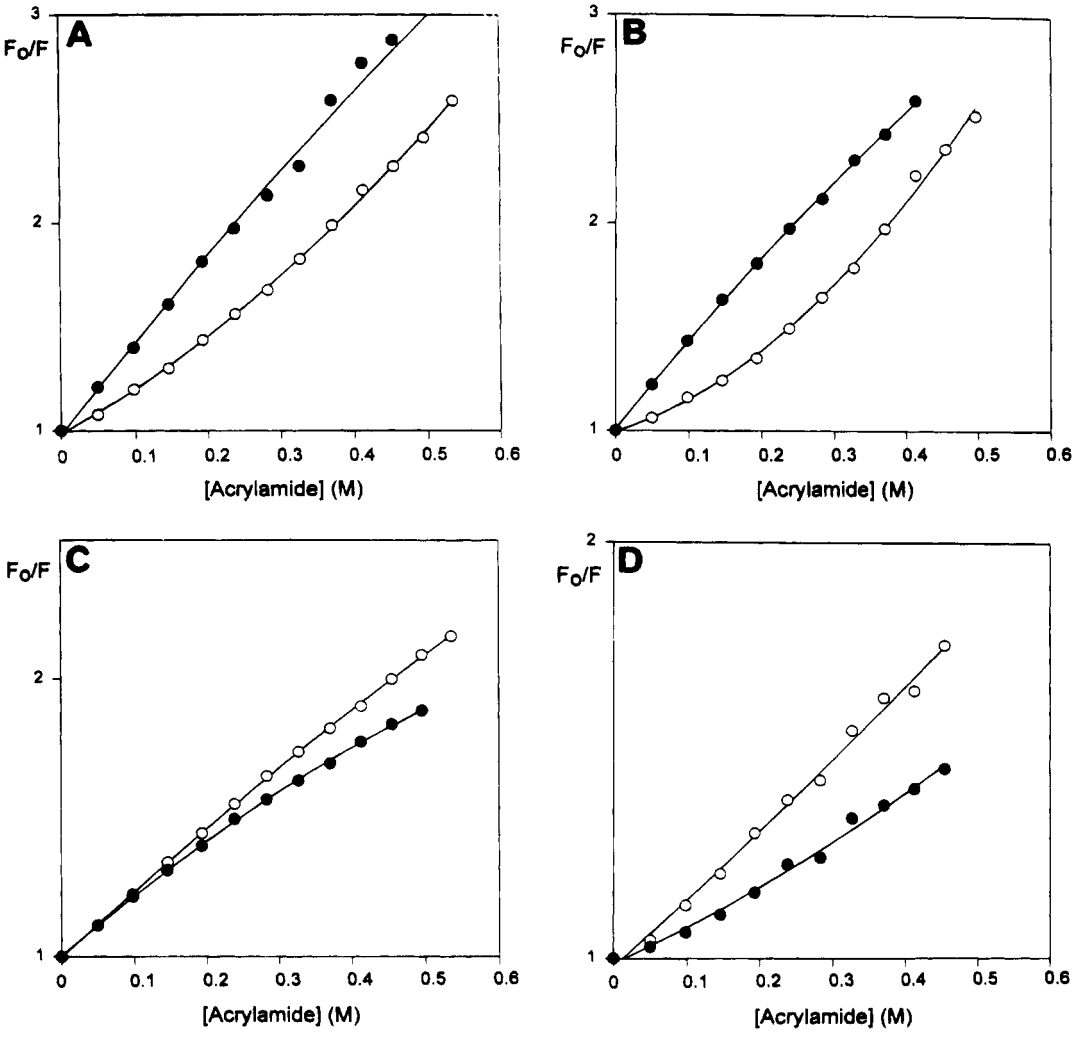

FIGURE 5: Stern–Volmer plots for the quenching of tryptophan fluorescence by acrylamide in wild-type hPAH (A) and double mutants containing only W120 (B), W187 (C), or W326 (D), in the absence (○) or in the presence of 1 mM phenylalanine (●). The conditions were as described in the legend to Figure 3.

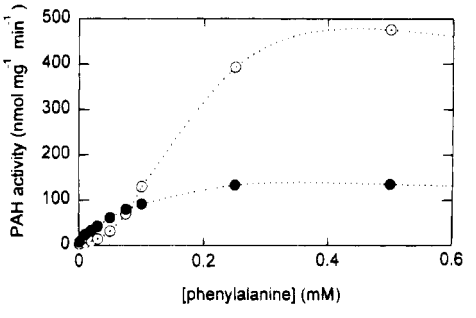

FIGURE 6: Effect of acrylamide on the activity of wild-type hPAH (factor Xa), using different concentrations of phenylalanine. The enzyme was incubated for 5 min under the conditions described under Materials and Methods, either in the absence (○) or in the presence of 1 M acrylamide (●) (added 5 min before the substrates).

Kaufman, 1994). Interestingly, the fusion protein was active also in this case, but the cleavage with factor Xa was unsuccessful. This may indicate subtle differences in the folding of the two fusion proteins, affecting the protease recognition site.

The activities of the fusion proteins were found to reflect the properties of the hPAH component of the fusion protein, relatively undisturbed by the presence of the fusion partner. Furthermore, the catalytic activities and regulatory properties of the multimutated hPAH fusion proteins seemed to be intermediate between the properties of the individually mutated proteins (Tables 3 and 4). The aggregation of the fusion proteins containing the W187F mutation and the purified hPAH mutants containing the W120F mutation shows that W120 and W187 are essential for the correct folding of the protein. Since all the mutants had high specific activities, whether as fusion proteins or as purified hPAH, the precipitation of the W120I and W120F mutants is more likely to be the result of an altered oligomeric state, favoring protein aggregation, than due to the biosynthesis of an "uncorrectly" folded protein. Since we found that the spectroscopic and kinetic properties of the wild-type hPAH were not influenced by the presence of small amounts of alternatively cleaved enzyme, the factor Xa construct was used for all the tryptophan mutants in this study.

The phenylalanine activation of PAH seems to be accompanied by an increased surface exposure of hydrophobic amino acids (Shiman et al., 1979), possible including tryptophans, and a shift from the dimeric to the tetrameric form of the enzyme (Døskeland et al., 1982). The W120I and W120F mutants had an increased affinity for phenylalanine and Hill coefficients (n_H) of only 1.3–1.7, compared to 2.0 and 2.4 for the wild-type fusion protein and purified hPAH, respectively. Thus, these mutants seemed to be in a "partially activated" state even without phenylalanine present,

and the aggregation of these mutants may reflect a conformational state related to that induced by phenylalanine binding. Interestingly, similar findings have been observed when hPAH containing a mutation associated with phenylketonuria (G46S) is expressed in the pMAL system (Eiken et al., 1995). These observations suggest that very small changes in the N-terminal part of the hPAH sequence can lead to dramatic changes in the aggregation state of the enzyme. No systematic study has been performed to determine if this aggregation is an irreversible process, or if it can be reversed by the inclusion of chaotropic agents, molecular chaperones, or other treatments. The fluorescence spectra of hPAH (excitation and emission maxima at 281 and 336.5 nm, respectively) are in excellent agreement with our previous data on rat and bovine PAH (278/335 nm) (Haavik et al., 1986), and close to the values (280−283/342 nm) reported by Koizumi et al. (1988) and Parniak et al. (1988). As shown in this study, the emission spectra are sensitive to experimental conditions, including buffer pH, providing an explanation for these small discrepancies. However, we have no explanation for the extremely blue-shifted emission spectra (maxima at 310−322 nm) reported for rat PAH by other investigators (Phillips et al., 1984; Marota & Shiman, 1984; Shiman et al., 1990).

As expected, the UV absorbance spectra of the different mutant proteins varied, as the number of tryptophan residues was varied between three and zero. The quantum yield of hPAH is in the range reported for most tryptophan-containing proteins (Burstein et al., 1973). Furthermore, fluorescence measurements on the mutants revealed that the quantum yields of the individual tryptophan residues in hPAH varied by a factor of more than 4, as reported for many other multi-tryptophan proteins. This study has demonstrated that most of the tryptophan fluorescence of hPAH is due to W120, and that this tryptophan residue also accounts for most of the shift in emission spectrum and increased quantum yield of the wild-type hPAH induced by phenylalanine binding. The quenching experiments show that W120, followed by W326, is the most surface-exposed of the tryptophan residues. This finding is consistent with a previous report on rat PAH, where they found partial fluorescence quenching by added KI (Phillips et al., 1984[2]).

The fluorescence of the different mutants also showed strikingly different responses to the addition of either phenylalanine or BH_4 (Tables 5−7). In principle, the lack of an effect of ligand binding on the tryptophan fluorescence of a mutant hPAH can be explained by several mechanisms. First, the mutant proteins may not be subject to the same conformational changes as the wild-type hPAH. Second, the protein may undergo the changes, but since it is lacking the spectroscopic probe(s), no effect is observed. Finally, a combination of these possibilities may be observed. In order to distinguish between the first two possibilities, the effect of phenylalanine on the enzyme activity was also investigated. While most of the mutants had maximal activities comparable to that of the wild-type hPAH, the effect on the phenylalanine activation process was striking (Tables 3 and 4). The concentrations of phenylalanine giving half-maximal velocity ($S_{0.5}$) varied with an order of magnitude between the different mutants, clearly showing that the W→F mutations did affect the substrate binding. However, even if the kinetic constants of the mutants were slightly altered compared to the wild-type hPAH, all the mutant proteins undergo the same conformational change when incubated with phenylalanine (Tables 3 and 4). Thus, the main conclusions, as reported here, regarding the assignment of the specific interactions of the different tryptophans were probably minimally affected by this. The striking effect of the W120I and W120F mutations on the aggregation state and the phenylalanine activation kinetics of hPAH is consistent with W120 being localized on the intersubunit interface of hPAH.

Several investigators have noticed that the rate of phenylalanine activation of rat PAH is increased at alkaline pH (Shiman & Gray, 1980; Parniak et al., 1988). Exposure of rat PAH to pH 9.5 also induces a shift of the fluorescence emission spectrum, very similar to that observed by phenylalanine binding (Parniak et al., 1988). This spectral shift, with an apparent pK_a value of about 8.0, was in the present study also demonstrated for the recombinant human enzyme, and extends the list of similarities in regulatory properties of PAH from the two species.

The fluorescence lifetimes of the tryptophans in rat PAH, in the absence and presence of phenylalanine, have previously been reported (Koizumi et al., 1988). In their analysis, they reported that the lifetimes of both of the two major components of the fluorescence increased on incubation with L-phenylalanine, and decreased in the presence BH_4, respectively. On the basis of the relative quantum yields, we assume that W120 accounts for at least some and perhaps all of the fluorescence component with the longest lifetime. The fluorescence lifetime of W120 is probably also increased in the presence of phenylalanine. However, since even single tryptophan proteins can exhibit nonexponential fluorescence decay curves with an apparent mixture of different lifetimes (Bajzer & Prendergast, 1993) and since Koizumi et al. (1988) were probably studying the tetrameric form of rat PAH, with a total of 12 tryptophan residues per enzyme molecule,[2] it is presently difficult to draw further conclusions from this work. With the availability of a series of single and multiple tryptophan PAH mutants, fluorescence lifetime studies should give information about the properties of all the individual tryptophan residues and their possible interactions. In conjunction with other experiments, this information can be used in order to determine which protein domains are involved in the substrate-induced conformational changes originally reported for rat PAH, and which we here have demonstrated for the recombinant human enzyme as well.

An important concern when studying protein structure/function by site-directed mutagenesis is that even a single "conservative" amino acid replacement may have multiple effects on the target protein. This is illustrated in the present study, where the W→F and W→I mutants had altered oligomeric structures, stabilities, and kinetic properties, in addition to the predicted spectroscopic changes. Since there seems to be a strong correlation between evolutionary amino acid conservation and functional consequences of their substitution (Markiewicz et al., 1994), it could have been predicted that the tryptophan residues of hPAH are of structural and functional significance. The characterization of the individual tryptophan residues of hPAH, as reported here, will further increase the usefulness of fluorescence spectroscopy in the studies of structure and function of this enzyme. Since hPAH catalyzes an important step in amino

acid catabolism, an increased knowledge about the structure and regulation of this enzyme is likely to be useful for the understanding of both normal amino acid metabolism and the pathogenesis of phenylketonuria/hyperphenylalaninemia.

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