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Effect of mutations at Cys^{237} on the activation state and activity of human phenylalanine hydroxylase

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Abstract Wild-type recombinant human phenylalanine hydroxylase (wt-hPAH) is activated about 1.5-fold by exposure to alkaline pH (pH 8.5–9.0). In order to study whereas this activation might be related to the activation of the rat enzyme by *N*-ethylamaleimide-modification of Cys^{237} [Gibbs and Benkovic (1991) *Biochemistry* 30, 6795], mutant proteins of hPAH with Cys^{237} changed to Ser (S) or Glu (D) have been prepared. The mutant forms have high specific activity at pH 7.0 and high affinity for L-Phe, notably for hPAH-C237D, which shows a 3-fold higher activity than L-Phe-activated wt-hPAH and it is not further activated by pre-incubation with L-Phe. Moreover, the emission maximum of the intrinsic fluorescence of hPAH-C237D (λ_{maxem} = 347 nm) resembles that of activated forms of wt-hPAH. However, the activity of this mutant at neutral pH is further activated by exposure to alkaline pH, indicating that activation of wt-hPAH by alkaline pH is not restricted to ionization of Cys^{237}.

Key words: Phenylalanine hydroxylase; Site-directed mutagenesis; Enzyme activation; Cooperativity; Tryptophan fluorescence

1. Introduction

Mammalian phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine, using tetrahydrobiopterin (BH_4) and dioxygen as additional substrates. PAH is activated by prior incubation with L-Phe [1] and we have postulated that this activation involves homotropic cooperative interactions between the catalytic sites of the tetrameric enzyme [2–4]. However, many aspects on the regulation (activation) of the enzyme by pre-incubation with L-Phe and by other physical and chemical procedures are still obscure [1,5]. Thus, the enzyme activated by prior incubation with L-Phe or by phosphorylation still expresses positive cooperativity for the substrate when the activity is assayed with BH_4 as cofactor, while other modes of activation of the enzyme, e.g. by incubation with lysolecithin, reaction with the sufhydryl reagent *N*-ethylamaleimide (NEM) or partial proteolysis, result in abolition of the cooperative binding of L-Phe [1,6].

Previous studies on the effect of pH on the activity of rat liver PAH (rPAH) have shown that exposure of the enzyme to alkaline pH facilitates the activation by the substrate [7,8] and also activates the enzyme per se without altering the positive cooperativity for L-Phe [8]. In addition, the exposure to alkaline pH and the incubation with L-Phe induce similar changes in the intrinsic fluorescence emission spectra in both rPAH [8] and recombinant human PAH (hPAH) [9]. In the absence of L-Phe exposure to alkaline pH promotes the dissociation of tetramers to dimers while L-Phe favors the formation of tetramers in the enzyme both at neutral and alkaline pH [4,10–12]. It has also been shown that the spectroscopical changes accompanying activation by L-Phe are also consistent with the ionization of a cysteine sulfhydryl group which becomes exposed upon activation [13]. Moreover, the treatment of rPAH with the sufhydryl reagent *N*-ethylamaleimide (NEM), reacting stoichiometrically per enzyme subunit, results both in activation [14] and in dissociation of tetramers into dimers [10]. By chemical modification of the rat enzyme Gibbs and Benkovic [15] determined that the modified sulfhydryl group was Cys^{236} (Cys^{237} if Met^1 is included). This residue is also conserved in human and mouse PAH [16] and in *Drosophila* [17].

In order to study the role of Cys^{237} on the activation of PAH and the relation between ionization of this residue and activation by alkaline pH, we have mutated Cys^{237} of recombinant hPAH. Cysteine was replaced either by serine (hPAH-C237S), as a non-ionizing residue, or by glutamic acid (hPAH-C237D), as a negatively charged residue at neutral pH. The activity and activation state, as well as some physical-chemical properties of the wild-type enzyme (wt-hPAH) and the mutants have been investigated as a function of pH.

2. Materials and methods

2.1. Materials

DNA primers for PCR and sequencing were synthesized on an Applied Biosystems (ABI) model 392 synthesizer. *Taq* DNA polymerase was from Boehringer-Mannheim. DNA sequencing was carried out by the Taq Dye DeoxyTerminator Cycle Sequencing Kit (ABI) using an automatic DNA sequencer (model 373A from ABI). Factor Xa was obtained from New England Biolabs.

2.2. Construction of vectors and site-directed mutagenesis

The subcloning of wt-hPAH cDNA into the pMAL-c2 vector (New England Biolabs) has recently been described [18]. The C237S and C237D mutations were introduced into the PAH cDNA (pMALPAH vector) by PCR-based site-directed mutagenesis as described [18] using the mutagenic primers A237S (5′-GAAATTGGAATCCTTAGC-3′) and A237D (5′- GAAATTGGAATCCTTGACAGTGC-3′), respectively. All positive clones were sequenced to verify the mutagenesis and to exclude other mutations due to Taq DNA polymerase misincorporations.

2.3. Expression and purification of recombinant wt-hPAH and its mutant forms

Expression of wt-hPAH, hPAH-C237S, hPAH-C237D and the

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Abbreviations: BH_4, (6*R*)-L-*erythro*-tetrahydrobiopterin; NEM, *N*-ethylmaleimide; PAH, phenylalanine hydroxylase; hPAH, human phenylalanine hydroxylase; rPAH, rat phenylalanine hydroxylase; MBP, maltose-binding protein; PKU, phenylketonuria; wt-hPAH, wild-type human phenylalanine hydroxylase

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truncated form hPAH-(Gly103–Gln428) in *Escherichia coli* (TB1), preparation of the cell lysates and purification of the fusion proteins by affinity chromatography on amylose resin followed by high-performance size exclusion chromatography was performed as described [4,11]. The tetrameric form of the fusion proteins was then cleaved by the restriction protease factor Xa. The standard conditions for cleavage were: 3 h at 4°C with fusion protein:factor Xa ratio 30:1. When indicated the isolated hPAH proteins were separated from MBP and factor Xa by size exclusion chromatography (see below), collected and concentrated by Immersible-CX-10 Ultrafilters (Millipore). The purity of both the fusion proteins and the isolated hPAH forms was determined by SDS-PAGE at 180 V in 10% (w/v) polyacrylamide gels which were stained by Coomassie brilliant blue.

Protein concentration was estimated by the Bradford method [19] using as standard proteins purified fusion protein and isolated hPAH, having the absorption coefficients A_{280nm} (1 mg ml^{-1} cm^{-1}) = 1.63 and 1.0, respectively [11].

Preparative and analytical size exclusion chromatography was performed at 4°C using a HiLoad Superdex 200 HR column (1.6×60 cm) as described [4,11].

2.4. Assay of phenylalanine hydroxylase activity

In order to study the effect of pH on the pre-incubation and/or assay of hPAH, different buffers of pH ranging from 5.5 to 9.0 were prepared: i.e. 250 mM K-Mes of pH 5.5, 6.0 and 6.5; 250 mM K-HEPES of pH 7.0, 7.5, 8.0 and 8.5 and 250 mM KCl/Na-borate (Clark and Lubs solutions) of pH 8.0, 8.5, 9.0 and 9.5. The actual pH value of the complete pre-incubation and assay mixtures was determined by direct measurements of the mixtures and were similar (±0.1 pH units) to that of the buffers. Unless otherwise indicated the enzyme samples used in kinetic studies were tetrameric form of the fusion protein MBP-hPAH after cleavage by factor Xa, both for wt-hPAH and the mutant forms. The enzyme was pre-incubated for 4 min at 25°C in a mixture containing 0.1 M buffer of pH as indicated and catalase (0.04 mg/ml), and in the presence or the absence of L-Phe, as indicated. Then, 100 mM ferrous ammonium sulfate was added and allowed to incubate for additional 1 min with the enzyme. An aliquot of this sample (1–2 μg hPAH) was then diluted 25-fold in the complete assay mix equilibrated at 25°C with catalase, iron and L-Phe (if required) at the same concentration as in pre-incubation and the reaction was started by the addition of BH_4 (or BH_4 and L-Phe, see text for details) containing DTT (5 mM final concentration). The reaction was stopped after 1 min (standard reaction time) by adding 1% (v/v) acetic acid in ethanol. L-Tyrosine was measured by HPLC and fluorimetric detection [11]. The amount of product was linear with the amount of enzyme added at the selected standard assay conditions. The kinetic parameters were calculated by non-linear regression analysis of the experimental data using the Hill equation and the Enzfitter program (Biosoft, Cambridge, UK).

2.5. Fluorescence measurements

Measurements were performed at 25°C in a medium containing 20 mM Na-Hepes, 0.2 M NaCl (pH 7.0) or 20 mM KCl/Na-borate, 0.2 M NaCl (pH 9.0), with or without 1 mM L-Phe, as indicated, and a protein concentration which gave an absorbance at 295 nm ($\lambda_{maxexcit}$) of approx. 0.02. A Perkin-Elmer LS-50 luminescence spectrometer with a constant-temperature cell holder and 1 cm path-length quartz cells with maximal stirring was used. The spectra were corrected for blank emission.

3. Results

3.1. Effect of pH on the stability, activity and activation of hPAH

The effect of pH on the stability of wt-hPAH was studied using tetrameric fusion protein MBP-hPAH after cleavage by factor Xa by incubating aliquots of the enzyme (0.1 mg/ml) in various buffers (0.1 M) of pH 5.5–9.5 (see Section 2 for details). After incubation for 30 min at 20°C at the various pH values, the activity was assayed at pH 7.0 and 25°C. At these conditions, the activity was stable at pH values from 5.0 to 9.5, unless for a 25% loss of activity at about pH 5.5, near the isoelectric point of the enzyme [11], and a 15% loss at pH 7.0–8.0 (results not shown). The activity of enzyme samples (1 mg/ml) after freezing in liquid nitrogen was found to be stable when kept at pH values from 6.0 to 7.0, while it decreased with increasing pH above this value, i.e. 20% remaining activity after freezing and thawing at pH 9.0. However, no activity was lost when the enzyme was frozen/thawed at pH 9.0 in the presence of 1 mM L-Phe.

The pH optimum for the BH_4-dependent activity of wt-hPAH varied with the activation state of the enzyme. Thus, the non-activated enzyme showed a pH optimum at about 8.5 with a broad peak around pH 7.0, while the pH optimum was about 7.5 and the activity at neutral pH was 2.5-fold higher for wt-hPAH activated by L-Phe (Fig. 1A). When assayed at pH 7.0 (standard assay conditions), the activity of wt-hPAH also depended on the pH during pre-incubation (Fig. 1B). Parniak et al. [8] had previously reported that rPAH was activated by exposure at alkaline pH in the absence of L-Phe, but if L-Phe was present during prior incubation, pH had no additional effect on the final activity at neutral pH. However, and as shown in Fig. 1B, if wt-hPAH was pre-incubated with L-Phe at pH 9.0, the activity measured at neutral pH was 50% higher than when prior incubation with l-Phe was carried out at pH 7.0. Moreover, the enzyme maintained the positive cooperativity for l-Phe when assayed at pH 7.0 after prior incubation at pH 9.0 (Hill coefficient $h = 2.0$ and $S_{0.5} = 117$ μM) (Table 1).

3.2. Oligomeric distribution and kinetic properties of the mutant proteins hPAH-C237S and hPAH-C237D

In the absence of l-Phe, exposure at alkaline pH promotes the dissociation of tetrameric forms of PAH to dimeric forms, while the enzyme remains tetrameric at pH ≥ 9.0 if l-Phe (1 mM) is present [4,12 and results not shown]. In order to test if ionization of the residue Cys 237 is involved in the dissociation of tetramers into dimers and/or the activation of wt-hPAH at alkaline pH (Fig. 1), we changed this cysteine

Table 1
Kinetic parameters of the wild-type and Cys^{237} mutant forms of hPAH

Enzyme	V_{max} (nmol Tyr/min/mg)	$S_{0.5}$(L-Phe) (μM)	h(L-Phe)
Wild-type	1000	236 ± 11	2.2
	(1450 ± 50)[a]	(117 ± 20)[a]	2.0[a]
C237S	1800 ± 32	178 ± 7	2.2
C237D	2780 ± 57	91 ± 20	1.7

Activity measured at pH 7.0 in cleaved tetrameric fusion protein. V_{max}, $S_{0.5}$(L-Phe) and the Hill coefficient h(L-Phe) were determined at 75 μM BH_4 and variable concentrations of L-Phe (0–5 mM). The enzymes were pre-incubated (5 min, 25°C, pH 7.0) with L-Phe at the same concentrations as in the assay. The numbers represent the values (±SEM) calculated by non-linear regression analysis of three independent experiments.
[a]Pre-incubation at pH 9.0; assay at pH 7.0

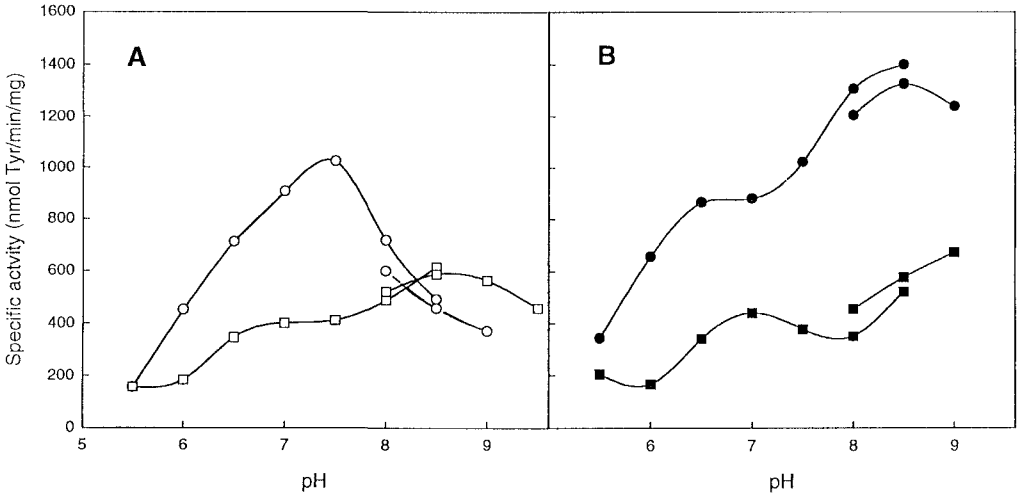

Fig. 1. The activity and activation of wt-hPAH as a function of the pH. A: Effect of pH on the activity of wt-hPAH: enzyme samples were pre-incubated (5 min, 25°C) at the indicated pH in the absence (□) or presence (○) of 1 mM L-Phe, and then assayed at the same pH with 1 mM L-Phe. B: Effect of varying pH during pre-incubation of hPAH on the activity at pH 7.0: enzyme samples were pre-incubated for 5 min at 25°C at the specified pH in the absence (■) or presence (●) of 1 mM L-Phe and then assayed with 1 mM L-Phe at pH 7.0. The various buffers used, of overlapping pH values, are indicated in Section 2.

residue to a serine (mutant hPAH-C237S) and to a glutamic acid (mutant hPAH-C237D). As shown in Fig. 2, these mutant forms were purified as fusion proteins to apparent homogeneity by amylose affinity chromatography, and further isolated by factor Xa cleavage and size exclusion chromatography. When analyzed by size exclusion chromatography at pH 7.0, the fusion proteins of both Cys 237 mutant forms consisted of tetrameric (about 90% of the total protein) and small amounts of aggregated and dimeric forms (results not shown). Moreover, the isolated hPAH-C237S and hPAH-C237D consisted mainly of tetramers, with the dimeric forms constituting ≤4% of the total hPAH protein for both mutants, as earlier obtained for wt-hPAH [4].

No significant differences were found between the pH dependence for activity of the Cys 237 mutant forms (results not shown) and wt-hPAH (Fig. 1A), and the mutants showed an optimum at about pH 8.5 with a lower peak around pH 7.0 when pre-incubated (5 min, 25°C) in the absence of l-Phe and at about pH 7.5 when pre-incubated with 1 mM l-Phe. As found for wt-hPAH, incubation at alkaline pH also stimulated the enzyme activity of both Cys 237 mutant forms measured at pH 7.0 (Fig. 3). Moreover, the activity of hPAH-C237S at neutral pH was increased when L-Phe was present during prior incubation in the whole pH range from 5.5 to 9.0 (Fig. 3A). In contrast, no further activation by L-Phe of the activity of hPAH-C237D was found, and in fact a small inhibition of the activity (≤20%) was measured when L-Phe was present during prior incubation at pH values from 6.0 to 7.0 and at about 8.0 (Fig. 3B). Steady-state kinetic studies showed that both Cys^{237} mutant forms, and notably hPAH-C237D, presented higher affinity for L-Phe and higher V_{max} than wt-hPAH, while the positive cooperativity for the substrate was conserved ($h > 1$) (Table 1). Moreover, both wt-hPAH and Cys^{237} mutants presented similar apparent K_m value for the cofactor BH_4 (30–40 μM), with no apparent cooperativity for its binding ($h = 1$).

The pH optima for activity and the effect of pH during prior incubation on the activity at neutral pH was also examined for a truncated form of the enzyme, hPAH(Gly103–Gln428), which is an 'activated' dimeric form with high affinity for L-Phe ($S_{0.5} = 60$ μM) and no positive cooperativity for the substrate ($h = 1$) [4]. This enzyme form presented very clear activity optima at pH 7.0 and about pH 7.5 for non-L-Phe-activated and L-Phe-activated samples, respectively. In contrast to the full-length forms of the enzyme investigated in this study, the activity at neutral pH of this truncated form was not activated by prior incubation at alkaline pH (results not shown).

3.3. Fluorescence emission spectra

The fluorescence excitation and emission spectra at neutral pH of both rPAH [13] and wt-hPAH [9] are typical of parti-

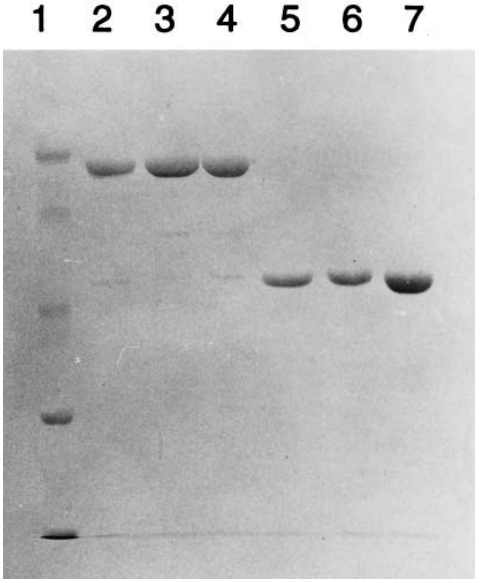

Fig. 2. Purification of wild-type and mutant forms (C237S and C237D) of hPAH. Lane 1: Low molecular mass standards; fusion proteins (MBP-hPAH), 94.2 kDa: wild-type hPAH (lane 2), hPAH-C237S (lane 3) and hPAH-C237D (lane 4); isolated hPAH proteins, 50 kDa, after cleavage by factor Xa and purification by size exclusion chromatography: wt-hPAH (lane 5), hPAH-C237S (lane 6) and hPAH-C237D (lane 7).

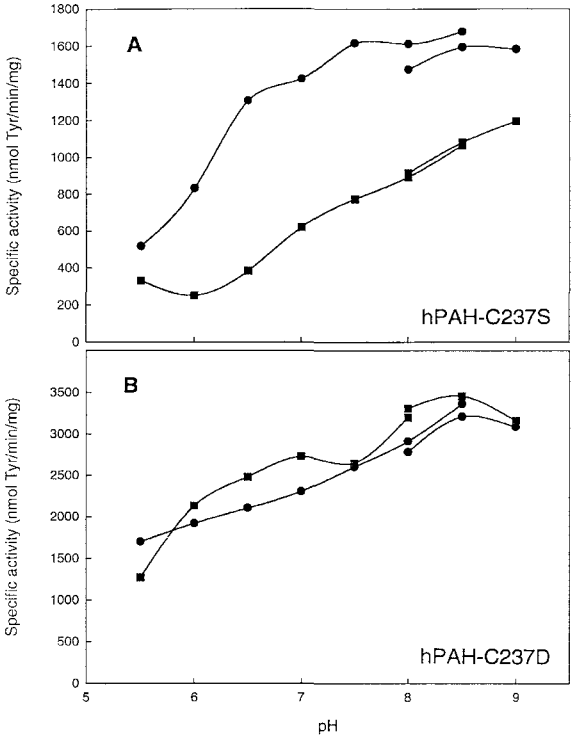

Fig. 3. The activation of hPAH-C237S (A) and hPAH-C237D (B) as a function of the pH. The enzyme samples were pre-incubated (5 min, 25°C) at the specified pH in the absence (■) or presence (●) of 1 mM L-Phe and then assayed with 1 mM L-Phe at pH 7.0. The various buffers used, of overlapping pH values, are indicated in Section 2.

ally solvent-exposed tryptophan residues ($\lambda_{maxexcit} = 281$ nm, $\lambda_{maxem} = 335–338$ nm), with highest contribution from Trp^{120} [9]. The λ_{maxem} shifts to about 347–348 nm and the fluorescence intensity increases on activation of the enzymes by incubation with L-Phe at neutral pH [9,13] and by exposure to alkaline pH [8,9]. For isolated hPAH-C237S a red shift of the λ_{maxem} and an increase in fluorescence intensity ($\approx 25\%$) are also induced on incubation with L-Phe at pH 7.0 (not shown) and on exposure to pH 9.0 (Fig. 4A), while on incubation with L-Phe at pH 9.0 an 11% increase of the fluorescence intensity ($\lambda_{maxem} = 347$ nm) is induced both for hPAH-C237S and wt-hPAH (not shown). However, hPAH-C237D has a $\lambda_{maxem} = 347$ nm at neutral pH and the fluorescence intensity further increased ($\approx 25\%$) at pH 9.0 (Fig. 4B), while on incubation with L-Phe a quenching of fluorescence intensity ($\approx 25\%$) was observed both at pH 7.0 and pH 9.0 (results not shown).

4. Discussion

The activation of wt-hPAH by L-Phe is accompanied by a change in the pH dependence of the BH_4-dependent activity of this enzyme. The pH optimum is about 8.5 for non-activated wt-hPAH, while maximal activity is obtained at pH 7.5 for L-Phe-activated enzyme. Similar results have been previously obtained with rPAH [8]. Other enzyme systems, e.g. aspartate transcarbamylase from *E. coli* [20,21] and *Hafnia alvei* aspartase [22] also reveal a shift of the pH optimum on activation of the enzymes by substrates and effectors which has been interpreted to result from changes in pK_a values of residues involved in substrate binding. These changes in pK_a values may be due to conformational changes undergone on activation, to direct interaction with the substrate, or both. For rPAH the shift in pH optimum has been interpreted as being the result of both an increased rate of activation by L-Phe at alkaline pH and to enzyme activation by alkaline pH per se through a conformational change involving the regulatory N-terminal domain [7,8]. Accordingly, 'activated' truncated forms lacking the N-terminal domain, which are not further activated by alkaline pH, show an activity maximum at about pH 7.0 even in the absence of L-Phe during preincubation [[8] and this work]. The finding that hPAH-C237D is further activated by alkaline pH and not by incubation with L-Phe at any pH value from 5.5 to 9.0 also indicates that alkaline pH activates wt-hPAH per se and that this activation is not only due to an increased rate of activation by L-Phe at alkaline pH compared with that at neutral pH [7]. Since the positive cooperativity of L-Phe binding is preserved at pH 9.0 [[8] and this work], the conformational change induced by alkaline pH does not seem to destroy the homotropic cooperative interactions and thus it seems to be qualitatively different from other modes of activation of the enzyme, e.g. incubation with the phospolipid lysolecithin [4,23,24].

As shown in this work, incorporation of a negative charge at residue 237 in hPAH, which is the one modified by derivatization and activation of the enzyme with NEM [15], also have activating effects on the enzyme. The mutant hPAH-C237D reveals 3- and 2-fold higher activity than L-Phe-activated wt-hPAH and N-terminal truncated forms, respectively, which is the highest specific activity measured so far for any mutant form of this enzyme. The mutant hPAH-C237D conserves the cooperative response to increasing concentration of L-Phe ($h = 1.7$) and its $\lambda_{maxem} = 347$ resembles that of wt-hPAH at alkaline pH [9], which might indicate that ionization of Cys^{237} was involved in the activation of the enzyme by alkaline pH. However, there are some findings indicating

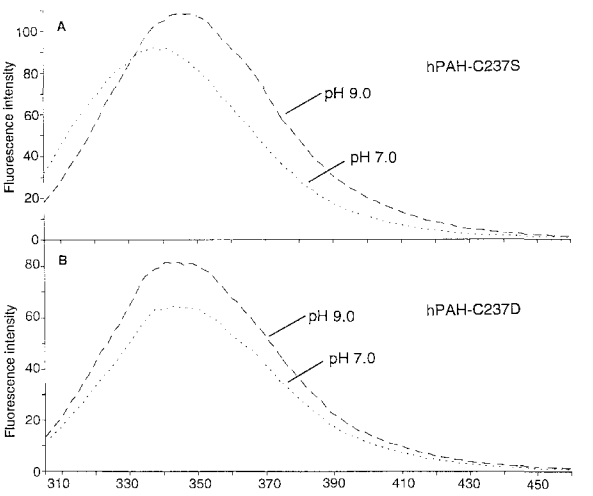

Fig. 4. Effect of pH on the fluorescence emission spectra ($\lambda_{exc} = 295$ nm) of isolated hPAH-C237S (A) and hPAH-C237D (B). The enzyme samples were diluted to a final concentration of 60 μg/ml in 20 mM Na-Hepes, 0.2 M NaCl, pH 7.0 (dotted line) and in 20 mM KCl/Na-borate, 0.2 M NaCl, pH 9.0 (broken line). The excitation and emission slits were 2.5 and 5.0, respectively. The emission maxima were: at pH 7.0, 337 nm (A) and 247 nm (B), and at pH 9.0, 347 nm (A and B).

that the C237D mutation and the incubation of wt-hPAH at alkaline pH induce different effects on the enzyme, i.e. (i) increasing pH induces the dissociation of tetramers into dimers [4,12] while hPAH-C237D was mainly tetrameric at neutral pH; (ii) exposure of wt-hPAH to alkaline pH and incubation with L-Phe seem to give additive effects on the activity and on the intensity of intrinsic fluorescence, while hPAH-C237D is not further activated by pre-incubation with L-Phe and its fluorescence intensity is quenched upon binding of the substrate, and (iii), and most conclusive, by incubation at alkaline pH hPAH-C237D is further activated and its fluorescence intensity increases at the same degree as wt-hPAH and hPAH-C237S. Thus, it seems that the conformational changes induced by ionization of Cys^{237} and exposure to alkaline pH are different and probably additive. Since the more conservative substitution Cys by Ser at position 237 also has some activating effects on the enzyme (higher activity and affinity for L-Phe than wt-hPAH), some of the effects of the C237D mutation on the enzyme seem to be due to a perturbation of the structure of the enzyme and not only to the incorporation of the negative charge at this position. Thus, mild denaturation of the enzyme has also been found to have an activating effect [25].

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