# Substrate-induced conformational transition in human phenylalanine hydroxylase as studied by surface plasmon resonance analyses: the effect of terminal deletions, substrate analogues and phosphorylation

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The optical biosensor technique, based on the surface plasmon resonance (SPR) phenomenon, was used for real-time measurements of the slow conformational transition (isomerization) which occurs in human phenylalanine hydroxylase (hPAH) on the binding/dissociation of L-phenylalanine (L-Phe). The binding to immobilized tetrameric wt-hPAH resulted in a time-dependent increase in the refractive index (up to approx. 3 min at 25 °C) with an end point of approx. 75 RU (resonance units)/(pmol subunit/mm²). By contrast, the contribution of binding the substrate (165 Da) to its catalytic core enzyme [ΔN(1–102)/ΔC(428–452)-hPAH] was only approx. 2 RU/(pmol subunit/mm²). The binding isotherm for tetrameric and dimeric wt-hPAH revealed a $[S]_{0.5}$-value of $98 \pm 7$ $\mu$M ($h = 1.0$) and $158 \pm 11$ $\mu$M, respectively, i.e. for the tetramer it is slightly lower than the value ($145 \pm 5$ $\mu$M) obtained for the co-operative binding ($h = 1.6 \pm 0.4$) of L-Phe as measured by the change in intrinsic tryptophan fluorescence. The responses obtained by SPR and intrinsic tryptophan fluorescence are both considered to be related to the slow reversible conformational transition which occurs in the enzyme upon L-Phe binding, i.e. by the transition from a low-activity state ('T-state') to a relaxed high-activity state ('R-state') characteristic of this hysteretic enzyme, however, the two methods reflect different elements of the transition. Studies on the N- and C-terminal truncated forms revealed that the N-terminal regulatory domain (residues 1–117) plus catalytic domain (residues 118–411) were required for the full signal amplitude of the SPR response. Both the on- and off-rates for the conformational transition were biphasic, which is interpreted in terms of a difference in the energy barrier and the rate by which the two domains (catalytic and regulatory) undergo a conformational change. The substrate analogue 3-(2-thienyl)-L-alanine revealed an SPR response comparable with that of L-Phe on binding to wild-type hPAH.

Key words: conformational transition, hysteresis, non-haem iron, phenylalanine hydroxylase, surface plasmon resonance (SPR).

## INTRODUCTION

Mammalian phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH, EC 1.14.16.1) is a pteridine-dependent non-haem-iron enzyme that catalyses the first and rate-limiting step in the metabolism of the essential amino acid L-phenylalanine (L-Phe) [1]. The activity of the enzyme is carefully controlled *in vivo* in order to maintain a constant, optimal blood concentration of the amino acid. Reduced or complete loss of catalytic activity represents the molecular basis for the autosomal recessive disorder hyperphenylalaninaemia/phenylketonuria (PKU) that can lead to severe mental retardation if not treated properly from birth [1]. More than 400 mutations have so far been identified at the human PAH (hPAH) locus and documented in a locus-specific mutation database (http://www.mcgill.ca/pahdb).

PAH exists in solution mainly as a homotetramer, but on increasing the pH the tetramer–dimer equilibrium shifts towards the dimeric form [2,3], whereas binding of L-Phe to rat PAH (rPAH) [2] and hPAH [3] favours the tetrameric form. Limited proteolysis, site-directed mutagenesis and crystal structure analyses have demonstrated that each subunit is organized into three main domains; a regulatory N-terminal domain (residues 2–117) containing the phosphorylation site (Ser-16), a catalytic core domain (residues 118–411) and a C-terminal tetramerization domain or motif (residues 412–452), responsible for the formation of tetramers (dimers of dimers) by a coiled-coil interaction of amphiphatic $\alpha$-helices (for review, see [4]). Tetrameric wild-type (wt)-PAH expresses positive kinetic co-operativity for L-Phe binding and activation [1,2,4]. Thus, in steady-state kinetics the wt tetramer shows a sigmoid relationship between the catalytic activity and the substrate concentration, reflecting a concentration dependent slow transition from a low-activity state ('T-state') with low affinity for L-Phe to a high-activity state ('R-state') with a higher affinity for L-Phe [5], characteristic for a hysteretic enzyme [6]. Several physico-chemical responses (conformational probes) have so far been used as manifestations of the substrate-induced conformational change in the tetrameric full-length PAH (Table 1). By intrinsic tryptophan fluorescence [11] and more recently by surface plasmon resonance (SPR) analyses [14], the conformational transition has been followed on the time scale of seconds to minutes, compatible with hysteresis [6]. The SPR technique is unique in that it can measure the reversible binding and related global conformational transition in real-time [14–19], whereas intrinsic tryptophan fluorescence only measures the on-rate for the perturbation (change in micro-environment) of a specific tryptophan residue (Trp-120 in hPAH) in each subunit of the tetramer [12].

Optical biosensors, based on the SPR phenomenon, have been widely used to monitor reversible biospecific interactions between

Abbreviations used: $BH_4$, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; 3D, three-dimensional; DTT, dithiothreitol; *h*, Hill coefficient; hPAH, human phenylalanine hydroxylase; L-Phe, L-phenylalanine; L-Tyr, L-tyrosine; PAH, phenylalanine hydroxylase; PKA, protein kinase A; PKU, phenylketonuria; rPAH, rat phenylalanine hydroxylase; RU, resonance units; SPR, surface plasmon resonance; $[S]_{0.5}$, substrate concentration yielding half-maximum saturation [the SPR response to the analyte is expressed in units of RU/(pmol subunit/mm²)]; wt, wild-type; wt-hPAH, wild-type human phenylalanine hydroxylase.

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**Table 1 Manifestations of the L-Phe induced conformational change in tetrameric full-length PAH as studied by different biochemical and biophysical methods**

| Manifestation of the conformational change | Analytical method | Species |
|---|---|---|
| Increase in surface hydrophobicity | Affinity chromatography | rat [7] |
| Increased limited proteolysis | Proteolysis | rat, human [8,9,10] |
| Red-shift and increased quantum yield of the intrinsic tryptophan fluorescence | Fluorescence | rat, human [11,12] |
| Perturbation of the ultraviolet difference spectrum | UV-spectroscopy | rat [11] |
| Changes in hydrodynamic properties | Dynamic light scattering | rat [2] |
| Kinetic cooperativity of L-Phe binding | Enzyme kinetics | rat, human [5,13] |
| Time-dependent $\Delta$RU response which was greater than expected from the molecular mass (165 Da) of L-Phe | Surface plasmon resonance (SPR) | human [14] |

macromolecules in real-time [20]. More recently, it has been demonstrated that this technique is also capable of detecting changes in the conformation of an immobilized protein [14–19], including the ligand induced conformational transition in maltose binding protein, the allosteric protein transglutaminase [17] and hPAH [14]. In the present study we have extended our SPR studies on hPAH to the N- and C-terminal truncated, catalytically active forms of the enzyme to obtain information on the structural domains involved and the dynamics of the substrate induced conformational transition in the wild-type tetrameric form. Furthermore, the effect of substrate analogues and phosphorylation of Ser16 by protein kinase A (PKA) have been studied in wt-hPAH.

## MATERIALS AND METHODS

### Materials

The pterin cofactor ($BH_4$) was purchased from Dr B. Schirck (Jona, Switzerland). The restriction protease factor Xa was obtained from Protein Engineering Technology (ApS, Aarhus, Denmark). The catalytic subunit of cAMP-dependent protein kinase was obtained from Boehringer Mannheim (Mannheim, Germany). The BiaCore X instrument, the CM5 sensor chip and the different reagents for the SPR analyses were all provided by BiaCore AB (Uppsala, Sweden).

### Expression and purification of wt-hPAH and its truncated forms

The recombinant wild-type and truncated forms $\Delta$N(1–102)-hPAH, $\Delta$N(1–102)/$\Delta$C(428–452)-hPAH and $\Delta$C(428–452)-hPAH were expressed as fusion proteins with the maltose binding protein as the fusion partner [MBP-$(pep)_{Xa}$-hPAH] in the pMAL system of *E. coli* and subsequently purified by affinity chromatography on an amylose resin [3,13]. The fusion proteins were cleaved by the restriction protease factor Xa and the cleaved enzymes purified to $> 98\,\%$ homogeneity using a HiLoad Superdex 200 HR column (1.6 cm $\times$ 60 cm), prepacked from Amersham Biosciences (Frieburg, Germany) as described previously [3]. The purity of the enzyme was analysed by SDS/PAGE (10 % gel) and stored in liquid nitrogen until used.

### Phosphorylation of wt-hPAH

In addition to cleavage of the fusion protein at the specific linker region, factor Xa also cleaves at an additional site (i.e. after Arg-13) which results in a mixture of full-length and truncated forms ($\Delta$N1-13) of isolated hPAH [21]. Arg-13 is part of the recognition site for the cAMP-dependent protein kinase, PKA, and the phosphorylation was therefore performed on the fusion protein prior to cleavage by the protease, which also prevents the non-specific cleavage after Arg-13 [21]. The phosphorylation reaction was carried out for 30 min at 30 °C in a reaction mixture consisting of 2 mM dithiothreitol (DTT), 60 mM magnesium acetate, 0.2 mM EDTA, 0.6 mM EGTA, 90 mM Hepes buffer (pH 7), 100 $\mu$M ATP and 100 nM catalytic subunit of PKA; the concentration of fusion protein was 5 mg/ml. Cleavage by factor Xa followed by size-exclusion chromatography was performed as described above for the non-phosphorylated form. The isolated phosphorylated hPAH was analysed by SDS/PAGE and the expected mobility shift was observed for the phosphorylated form as compared with the non-phosphorylated wild-type [21].

### SPR analyses

The interaction analyses between immobilized hPAH and its substrates (analytes) were performed using the dual-flow-cell BiaCore X instrument, which is based on the optical phenomenon SPR [20]. Immobilization of the enzyme (wt-hPAH and the different truncated forms) to the hydrophilic carboxymethylated dextran matrix of the sensor chip (CM5) was performed by the standard primary amine coupling reaction, as described previously for tyrosine hydroxylase [22]. The enzyme to be covalently bound to the matrix was diluted in 10 mM sodium acetate buffer, pH 5.4, to a final concentration of 0.23 or 0.37 mg/ml, a pH which stabilizes the tetrameric form. An increase in $\Delta$RU (change in resonance units) of 15000–26000 RU was obtained, depending on the form of the enzyme immobilized to the matrix, consistent with about 15–26 ng of immobilized protein/mm$^2$ [14]. In the initial experiments egg-white lysozyme was immobilized on the reference surface to a $\Delta$RU of about 8300, in order to correct for any unspecific binding of the analyte together with changes in the bulk refractive index [14,22]. After the coupling procedure, 70 $\mu$l of 10 mM DTT was passed through both flow cells in order to reduce the time required to reach a stable baseline (empirical observation [22]). Further equilibration of the baseline was obtained by a continuous flow of HBS running buffer [10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.005 % (v/v) surfactant P20, pH 7.4] for 1–2 h, and a constant baseline drift of about $-0.05$ RU/s was then obtained for the tetrameric wt-hPAH. All biosensor analyses were performed with HBS running buffer at a constant flow of 5 $\mu$l/min and at a temperature of 25 °C, unless otherwise indicated. The immobilized enzyme was stable for at least 6 h at 25 °C, based on the reproducibility of the SPR response to added analyte within this time period. The sensorgram from each injection was obtained by automatic correction for non-specific bulk refractive index effects by subtracting the signal in the reference cell from that obtained in the sample cell.

The effects of different analytes (L-Phe and substrate analogues) on the refractive index of immobilized hPAH and its truncated forms were studied by injecting different concentrations of the

analytes, diluted in HBS buffer (pH 7.4) over the reaction surface.

The SPR response and the apparent overall half-time for the on- and off-rates observed for the reversible analyte binding were measured directly from the sensorgrams, using the cursor guided reading of the X- and Y-co-ordinates. From the binding isotherm the concentration of the analyte at half maximum response ($[S]_{0.5}$) was calculated by non-linear regression analysis using the software program EnzFitter for Windows (version 2.0.3) from Biosoft (1999). The SPR response of substrate binding to the different molecular forms of hPAH was related to the calculated amount of bound enzyme by assuming that 1000 RU corresponds to 1 ng of protein bound/mm$^2$ [20], and expressed as $\Delta$RU/(pmol subunit/mm$^2$).

### Tryptophan fluorescence

Intrinsic tryptophan fluorescence measurements of tetrameric wt-hPAH and dimeric $\Delta$C(428–452)-hPAH were performed at 25 °C in a buffer containing 20 mM Hepes and 0.2 M NaCl, pH 7.0, and a protein concentration of 0.02 mg/ml. The excitation and emission wavelengths used were 295 and 358 nm, respectively [12]. Increasing concentrations of L-Phe or D-Phe were added to the incubation mixture every third minute, and the change in fluorescence intensity ($\Delta$F) was measured as a function of the concentration of added L-Phe or D-Phe. The binding isotherm for L-Phe was obtained by subtracting the response to added D-Phe from the response to added L-Phe in order to correct for a small unspecific signal, including baseline drift.

### Activity measurements

The catalytic activity of wt-hPAH and its truncated forms was measured at 25 °C in a reaction mixture containing about 1 $\mu$M subunit of hPAH, 0.5 mg/ml BSA, 1 mM DTT, 0.5 mg/ml catalase, 20 $\mu$M ferrous ammonium sulphate in 100 mM Hepes buffer, pH 7, and with varying concentration of L-Phe (pre-incubation for 4 min). The reaction was started by adding 75 $\mu$M $BH_4$ and the amount of L-Tyr formed after the selected reaction time (1 min) was measured by HPLC [23].

## RESULTS

### Expression of wild-type and truncated forms of hPAH

The expression of the wild-type and three truncated forms of hPAH as fusion proteins in *E. coli*, using the pMAL vector system [3,13,24], resulted in high yields of purified functionally active enzyme forms. Stable oligomeric forms of wt-hPAH and the truncated forms $\Delta$N(1–102)-hPAH, $\Delta$C(428–452)-hPAH and $\Delta$N(1–102)/$\Delta$C(428–452)-hPAH, were isolated by size-

**Table 2 Steady-state kinetic constants of wild-type and different truncated forms of hPAH**

Data are means ± S.E.M.

| Enzyme | $V_{max}$ (nmol/min/mg) | $[S]_{0.5}$ (L-Phe) ($\mu$M) | Hill coefficient (L-Phe) |
|---|---|---|---|
| Wild-type tetramer | 4946 ± 281 | 135 ± 11 | 1.9 ± 0.1 |
| Wild-type dimer | 1703 ± 58 | 324 ± 11 | 0.8 ± 0.03 |
| $\Delta$N(1–102) tetramer | 6481 ± 432 | 78 ± 11 | 1.1 ± 0.1 |
| $\Delta$N(1–102)/$\Delta$C(428–452) dimer | 8318 ± 573 | 75 ± 10 | 1.3 ± 0.1[a] |
| $\Delta$C(428–452) dimer | 2224 ± 187 | 845 ± 169 | 1.1 ± 0.3 |

[a] Calculated from steady-state kinetic data with pronounced substrate inhibition, by non-linear regression analysis [25].

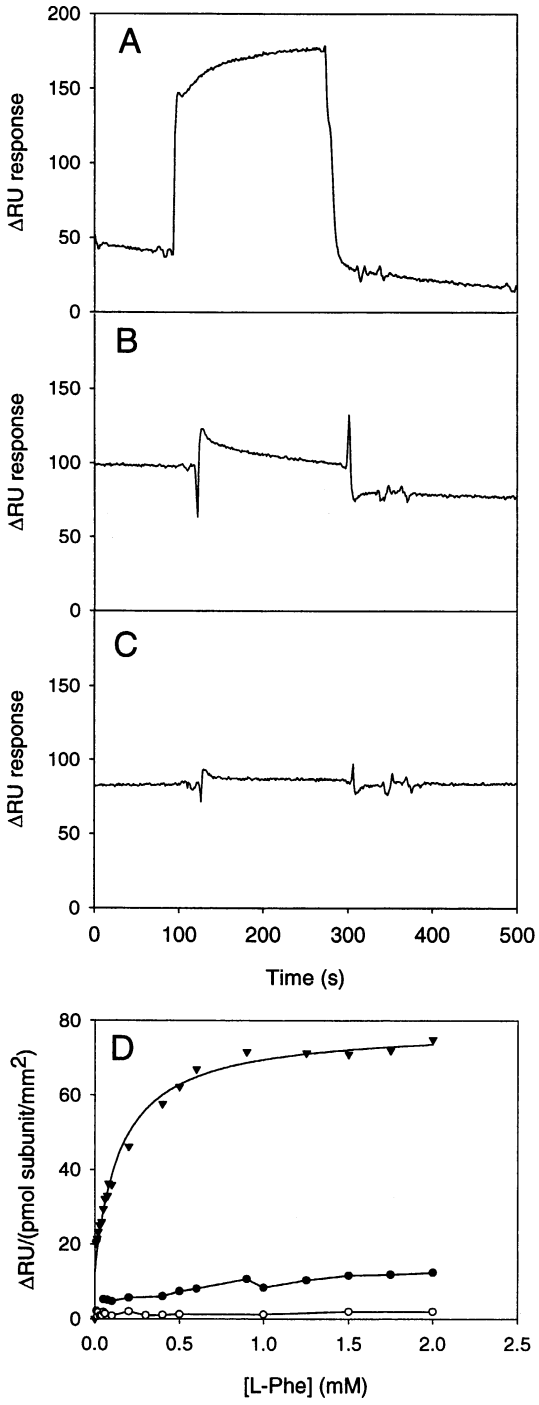


**Figure 1 Reversible binding of L-Phe to different molecular forms of hPAH as determined by SPR analyses**

Three sensorgrams display the reversible binding of L-Phe to (**A**) tetrameric wt-hPAH, (**B**) to the N-terminal truncated form $\Delta$N(1–102)-hPAH, and (**C**) to the double truncated form $\Delta$N(1–102)/$\Delta$C(428–452)-hPAH. L-Phe (2 mM) in HBS buffer was injected over the immobilized enzyme forms. (**D**) Effect of L-Phe concentration on the steady-state equilibrium SPR response of wt-hPAH (▼), $\Delta$N(1–102)-hPAH (●) and $\Delta$N(1–102)/$\Delta$C(428–452)-hPAH (○). Immobilization of wt-hPAH resulted in a SPR response of 26500 RU, consistent with 0.127 pmol enzyme/mm$^2$ (0.51 pmol subunit/mm$^2$); immobilization of $\Delta$N(1–102)-hPAH resulted in a response of 17300 RU, consistent with 0.108 pmol enzyme/mm$^2$ (0.432 pmol subunit/mm$^2$) and immobilization of $\Delta$N(1–102)/$\Delta$C(428–452)-hPAH resulted in a response of 14980 RU, consistent with 0.192 pmol enzyme/mm$^2$ (0.384 pmol subunit/mm$^2$). For calculations, see the Materials and methods section. In all three experiments immobilized lysozyme (8300 RU) was present in the reference cell.

**Table 3 Kinetic parameters for the substrate-induced changes in refractive index (ΔRU) of immobilized wt-hPAH (tetramer and dimer) and the C-terminal truncated form ΔC(428–452)-hPAH as measured by SPR**

The $[S]_{0.5}$-values represent the concentration of L-Phe which gave half-maximum steady-state equilibrium SPR response. The apparent $t_{1/2,obs}$-values for the on- and off-rates represent the half-time for the overall ΔRU response on injection of 2 mM L-Phe or 3-(2-thienyl)-L-alanine as calculated directly from the sensorgrams. All analyses were performed at 25 °C, unless otherwise indicated and data are mean ± S.E.M. For experimental details, see the Materials and methods section. n.d., not determined.

| hPAH | Analyte | $[S]_{0.5}$ (μM) | $t_{1/2,obs}$-on (s) | $t_{1/2,obs}$-off (s) |
|---|---|---|---|---|
| Wild-type tetramer | L-Phe | 97 ± 6 | 6 ± 2[a] | 9 ± 1[a] |
| Wild-type tetramer, at 10 °C | L-Phe | n.d. | 21 | 17 |
| Wild-type dimer | L-Phe | 158 ± 11 | n.d. | 4.5 |
| ΔC(428–452) dimer | L-Phe | 756 ± 64 | n.d. | 9.5 |
| Wild-type tetramer | 3-(2-thienyl)-L-alanine | 251 ± 35 | 3.5 | 5.5 |
| Wild-type tetramer, phosphorylated | L-Phe | 98 ± 7 | 5.5 ± 4[b] | 8 ± 1.5[b] |

[a] The $t_{1/2,obs}$-values for the on- and off-rate are average values from five different injections of L-Phe.
[b] The $t_{1/2,obs}$-values for the on- and off-rate are average values from four different injections of L-Phe.

exclusion chromatography to yield a purity of > 98 % on SDS/PAGE [3,13,24]. The wt-hPAH was recovered mainly in the tetrameric form (209 kDa), with a small proportion of dimeric hPAH, both isolated as stable chromatographic species at 4 °C [24]. ΔN(1–102)-hPAH (160 kDa) was recovered in a tetrameric form, and the truncated forms ΔC(428–452)-hPAH (98 kDa) and ΔN(1–102)/ΔC(428–452)-hPAH (76 kDa) were both dimers. Their catalytic properties are summarized in Table 2.

## SPR analysis of wild-type and N- and C-terminal truncated forms of hPAH

Immobilization of tetrameric wt-hPAH typically resulted in a ΔRU of about 25000 RU, corresponding to about 0.48 pmol subunit/mm². The concentrations of the different truncated forms were adjusted to result in similar amounts of immobilized subunit; ΔN(1–102)-hPAH gave a ΔRU value of about 17300 RU (0.43 pmol subunit/mm²), ΔN(1–102)/ΔC(428–452)-hPAH of about 15000 RU (0.38 pmol subunit/mm²) and ΔC(428–452)-hPAH of about 15000 RU (0.32 pmol subunit/mm²). Lysozyme (14.6 kDa) was initially immobilized to the reference cell to serve as a control surface as described in the Materials and methods section. The pH (5.4) selected for immobilization favours the formation of small amounts of higher oligomeric forms (results not shown), which may cause the observed small drift in the baseline (about −0.05 RU/s) for tetrameric wt-hPAH [14]. However, during a total titration period of 90 min, this drift represents only 1 % of the RU value measured for the amount of immobilized tetrameric wt-hPAH. For the truncated forms the drift was even smaller.

L-Phe is a low-molecular-mass compound (165 Da), and its binding to immobilized hPAH was expected to be at the limit of detection [20]. This was confirmed when L-Phe was injected over the sensor chip surface with the immobilized catalytically active core enzyme ΔN(1–102)/ΔC(428–452)-hPAH (Figures 1C and 1D), which resulted in a ΔRU response of only ~ 2 RU/(pmol subunit/mm²) at saturation. By contrast, with immobilized tetrameric wt-hPAH, a time-dependent increase in RU and a much higher SPR response at the end point (reached after ~ 3 min) was observed in the sensorgram (Figure 1A). This was consistent with our previous finding, and interpreted in terms of a global conformational change, which occurs in wt-hPAH on L-Phe binding [14]. Saturation was reached at approx. 2 mM with a ΔRU value of ~ 75 RU/(pmol subunit/mm²), and as seen from Figure 1(D) the steady-state binding isotherm revealed a hyperbolic concentration-dependence with a half-maximum ΔRU response ($[S]_{0.5}$) of 97 ± 6 μM (Table 3). By comparison, a value of 158 ± 11 μM was obtained for the wt-hPAH dimer together with a slightly lower ΔRU response at saturation (Figure 2A and Table 3). Interestingly, the reversible conformational transition in tetrameric wt-hPAH revealed a biphasic time-course for both the on- and off-responses, with an apparent overall half-time of 6 ± 2 s and 9 ± 1 s at 25 °C, respectively (Figures 3A and 3B and Table 3). It should be noted, however, that the small initial changes in the bulk refractive index, which in some experiments overlaps with the conformational transition during the first few seconds after the injection of L-Phe, may have some effect on the estimated half-time in the different experiments. The initial on- and off-rates were rapid, followed by a slower second phase (Figure 3), and were most clearly observed in the wt-hPAH at 10 °C, where a pronounced retardation of the second phase was observed (Figures 3C and 3D), and the apparent overall half-time for the on- and off-response was measured to be 21 s and 17 s, respectively (Table 3). Furthermore, the wild-type dimer revealed the same type of biphasic pattern for the transition at 25 °C (results not shown). At temperatures above 30 °C the biphasicity was not detectable (results not shown). The experimental error for the SPR response in replicate injections of the analyte was found to be in the range 1–4 % in three independent titration experiments.

## The SPR response on binding L-Phe to different N- and C-terminal-truncated forms of hPAH

The L-Phe binding to the three catalytically active truncated forms of hPAH [13] was studied in order to obtain information on the domains involved in the conformational transition in wt-hPAH. The crystal structures of the three truncated forms (ΔN(1–102)-hPAH, ΔN(1–102)/ΔC(428–452)-hPAH and ΔC(428–452)-hPAH) have been determined [4,26–28], and superposition of the three-dimensional (3D) structures have revealed the same overall fold of the catalytic domain in all the structures, with low root-mean-square deviation values. The binding of L-Phe at saturating concentrations, as determined from steady-state enzyme kinetics (Table 2), to immobilized tetrameric ΔN(1–102)-hPAH and dimeric ΔN(1–102)/ΔC(428–452)-hPAH had only a minor effect on their SPR responses, with ΔRU/(pmol subunit/mm²) at saturation values of ~ 14 and ~ 2, respectively (Figures 1B, 1C and 1D). For both enzyme forms the time-course revealed a 'square-wave' pattern, i.e. the full SPR response was reached within seconds and it returned rapidly to the baseline level at the end of the injection cycle, as expected from simple binding of a low-molecular-mass analyte [14,22]. On the other hand, the dimeric ΔC(428–452)-hPAH revealed a time-dependent SPR response on L-Phe binding similar to that observed for the wt-hPAH, with an estimated overall

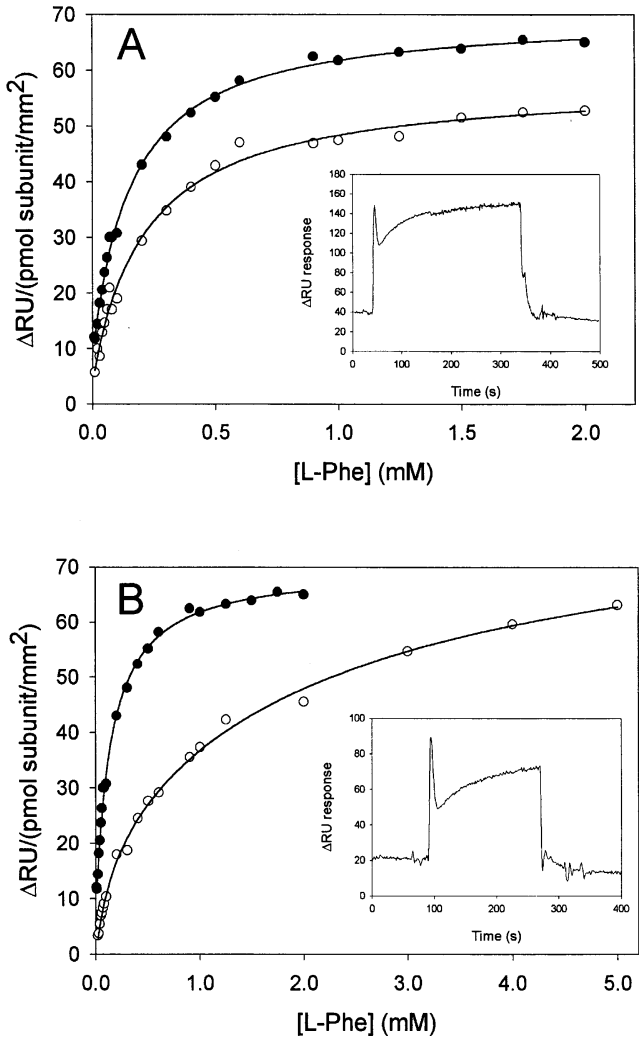


**Figure 2 Comparison of the effect of L-Phe concentration on the SPR response of immobilized wt-hPAH, tetramer and dimer, and the truncated form ΔC(428–452)-hPAH**

(**A**) Effect of increasing L-Phe concentrations on the SPR response of wild-type tetramer (●) and dimer (○). Immobilization of the dimer resulted in a response of 28350 RU, consistent with 0.271 pmol enzyme/mm$^2$ (0.542 pmol subunit/mm$^2$). (**B**) Effect of L-Phe concentration on the SPR response of the wild-type tetramer (●) and ΔC(428–452)-hPAH (○). Immobilization of ΔC(428–452)-hPAH resulted in a response of 14750 RU, consistent with 0.162 pmol enzyme/mm$^2$ (0.324 pmol subunit/mm$^2$). The response isotherm for the wild-type tetramer in both panels represents the average of two separate titration experiments with a mean basal ΔRU-value of 25090, consistent with 0.12 pmol enzyme/mm$^2$ (0.48 pmol subunit/mm$^2$). The truncated form ΔN(1–102)/ΔC(428–452)-hPAH was present in the reference cell (~ 0.4 pmol subunit/mm$^2$). For calculations, see the Materials and methods section. A sensorgram showing the effect of 2 mM L-Phe on immobilized wild-type dimer and ΔC(428–452)-hPAH is shown as an inset in Panel (**A**) and (**B**), respectively.

half-time for the off-response of 9.5 s. However, a higher concentration (> 5 mM) of the substrate was required to reach saturation (Figure 2B). Thus, the $[S]_{0.5}$-value for L-Phe was about 8-fold higher ($756 \pm 64$ $\mu$M) than that of the wt-hPAH ($97 \pm 6$ $\mu$M) (Table 3), i.e. similar to the values obtained from the binding isotherm determined by intrinsic tryptophan fluorescence measurements ($817 \pm 179$ $\mu$M) (Table 4) and steady-state activity measurements ($845 \pm 169$ $\mu$M) (Table 2).

In order to study the conformational transitions in wt-hPAH more selectively, the truncated form ΔN(1–102)/ΔC(428–452)-hPAH was used as the reference surface in the following studies, thereby eliminating the small contribution to the SPR response of binding the 165 Da L-Phe analyte to the active site.

### The effect of substrate analogues

It has previously been reported that D-Phe (at high concentrations) and the substrate analogue 3-(2-thienyl)-L-alanine are able to activate rPAH [29] and in the present study this was further analysed by passing these compounds over immobilized wt-hPAH (Figure 4). A small 'square-wave' type of response, typical for the binding of low-molecular-mass analytes, was observed for the injection of 2 mM D-Phe, with an increase in SPR signal (ΔRU) of only approx. 13 RU/(pmol subunit/mm$^2$) (Figure 4B) as compared with the time-dependent ΔRU of 75 RU/(pmol subunit/mm$^2$) for L-Phe. By contrast, 3-(2-thienyl)-L-alanine gave a time-dependent SPR response comparable with that of L-Phe in terms of both the time-course for the transition and the maximum ΔRU value (Figures 4 and 5), consistent with previous findings for the rat liver enzyme regarding its catalytic activation by the analogue [5,29]. However, the $[S]_{0.5}$-value ($251 \pm 35$ $\mu$M) for 3-(2-thienyl)-L-alanine was slightly higher than that for L-Phe (Table 3).

### The effect of phosphorylation

hPAH (and rPAH) are both phosphorylated at Ser-16 by PKA, which sensitizes the enzyme towards L-Phe activation, i.e. slightly lowers the concentration of L-Phe required for catalytic activation [21,23], and the specific activity of wt-hPAH increases 1.6-fold [21]. However, no significant difference could be observed between the conformational transition in the phosphorylated and the non-phosphorylated forms of wt-hPAH when analysed by SPR (Figure 6); the same end point and $[S]_{0.5}$-value of the binding isotherm for L-Phe was obtained (Figure 6 and Table 3).

### Intrinsic tryptophan fluorescence measurements

The conformational transition observed in tetrameric wt-hPAH on L-Phe binding has been reported to be associated with changes in the intrinsic tryptophan fluorescence spectrum of the tetrameric enzyme (see Table 1). On L-Phe binding its emission spectrum is red-shifted by about 10 nm and the quantum yield is increased due to a displacement of Trp-120 to a more hydrophilic environment [12]. From Figure 7(A) it is seen that the change in intrinsic tryptophan fluorescence of tetrameric wt-hPAH displayed a sigmoidal binding isotherm ($h = 1.6 \pm 0.4$) of L-Phe binding, with a $[S]_{0.5}$-value of $145 \pm 5$ $\mu$M as compared with the non-co-operative SPR binding isotherm ($[S]_{0.5} = 97 \pm 6$ $\mu$M) (Figure 7 and Tables 3 and 4). The progress curve (results not shown) with an apparent half-time for the on-rate of about 7 s, was also similar to that ($6 \pm 2$ s) obtained in the SPR analysis (Tables 3 and 4). By contrast, almost no change in the intrinsic tryptophan fluorescence spectrum was observed with D-Phe (in the same concentration range) as the analyte (results not shown). Moreover, the dimeric truncated form ΔC(428–452)-hPAH revealed a hyperbolic binding isotherm for L-Phe, both by intrinsic tryptophan fluorescence measurements and by SPR analysis (Figure 8), with $[S]_{0.5}$-values of $817 \pm 179$ and $756 \pm 64$ $\mu$M, respectively (Tables 3 and 4). The apparent half-time for this transition, as measured by intrinsic tryptophan fluorescence, was 7.5 s for the on-rate, i.e. similar to the value observed for the wild-type tetramer (Table 4).

## DISCUSSION

Based on a number of independent criteria (Table 1) it is generally accepted that mammalian PAH exists in at least two

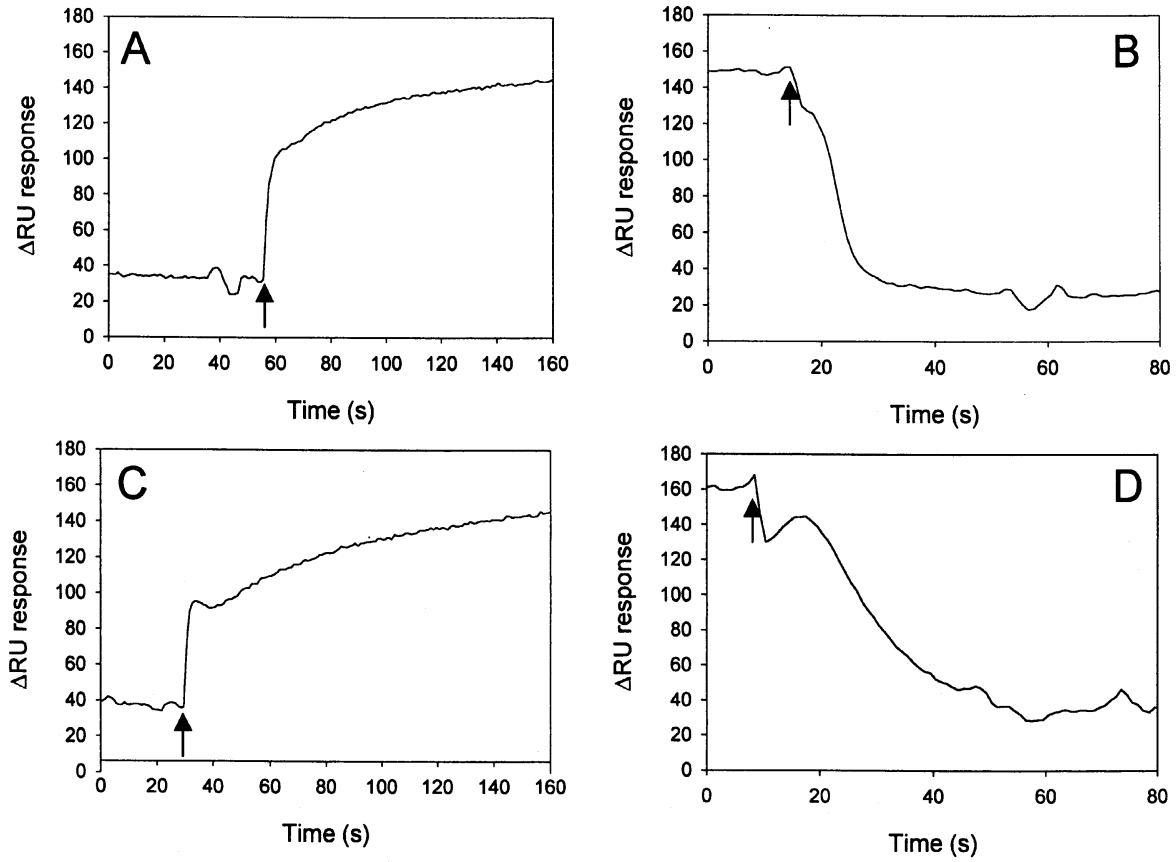


**Figure 3 Sensorgrams showing the on- and off-SPR responses to L-Phe injection over immobilized tetrameric wt-hPAH at different temperatures**

Sensorgrams (**A**) and (**B**) show the on- and off-responses, respectively, on injection of 2 mM L-Phe diluted in HBS buffer over the sensor chip surface containing the immobilized tetrameric wt-hPAH at 25 ± 0.01 °C. Sensorgrams (**C**) and (**D**) show the on- and off-responses at 10 ± 0.01 °C on injection of the same concentration of L-Phe. For experimental conditions, see the legend to Figure 1. ΔN(1–102)/ΔC(428–452)-hPAH was immobilized on the chip of the reference cell (~ 0.4 pmol subunit/mm$^2$). The arrows indicate the start or the end of the analyte injection cycle.

**Table 4 Parameters for the equilibrium binding of L-Phe to wt-hPAH and ΔC(428–452)-hPAH as determined by the change in intrinsic tryptophan fluorescence**

The $[S]_{0.5}$-values were calculated from the steady-state equilibrium response (ΔF) and represent the concentration of L-Phe which gave half-maximum response. $[S]_{0.5}$-values and the Hill coefficient were calculated by non-linear regression analyses as described in the Materials and methods section (mean ± S.E.M.).

| hPAH | $[S]_{0.5}$ (L-Phe) ($\mu$M) | Hill coefficient (L-Phe) | $t_{1/2,obs}$-on (s) |
|---|---|---|---|
| Wild-type tetramer | 145 ± 5 | 1.60 ± 0.4 | 7 |
| ΔC(428–452) dimer | 817 ± 179 | Non-co-operative binding | 7.5 |

different activity and conformational states, and that the enzyme undergoes a large conformational change (isomerization) on binding the substrate L-Phe. Thus, the catalytic activation of tetrameric mammalian PAH by its substrate can be represented by the following main equilibria:

$$\text{PAH} + \text{Phe} \underset{-k_1}{\overset{k_1}{\leftrightarrow}} \text{PAH·Phe} \underset{-k_2}{\overset{k_2}{\leftrightarrow}} \text{PAH*·Phe} \quad (1)$$

where PAH represents the free, low-activity state (designated the 'T-state') of the enzyme, PAH·Phe the binary enzyme-substrate complex and PAH*·Phe the binary complex of the relaxed high-activity state (designated the 'R-state') of the primed enzyme (PAH·Phe) in which a relatively slow conformational transition (relaxation) has occurred [14]. For the recombinant wt-hPAH the rate constant $k_1 \gg k_2$. In the present study our extended conformational studies by SPR analyses have given further information on (i) the structural basis for the reversible conformational transition, (ii) the dynamic aspects of the process, (iii) the regulatory substrate-binding site, and (iv) substrate analogues which induce the conformational change.

## The structural basis for the hysteretic conformational transition in hPAH

Crystal structure analyses of hPAH and rPAH (for review, see [4]) have revealed that each subunit of the homotetrameric enzyme contains three main structural and functional domains, i.e. a regulatory (residues 1–117), a catalytic (residues 118–411) and a tetramerization (residues 412–452) domain. Dimerization of two monomers is mediated through two symmetrically related hydrogen-bonded loops, residues 414–420 in the two subunits, with a buried surface area of 440 Å$^2$ [26], and through a relatively polar interface contributed by the regulatory domain in one subunit and the catalytic domain in the other subunit, with a buried surface area of 3560 Å$^2$ [27]. The tetramer (dimer of dimers) is formed primarily by interaction of the coiled-coil motif in the tetramerization domains of the four subunits [28]. Since

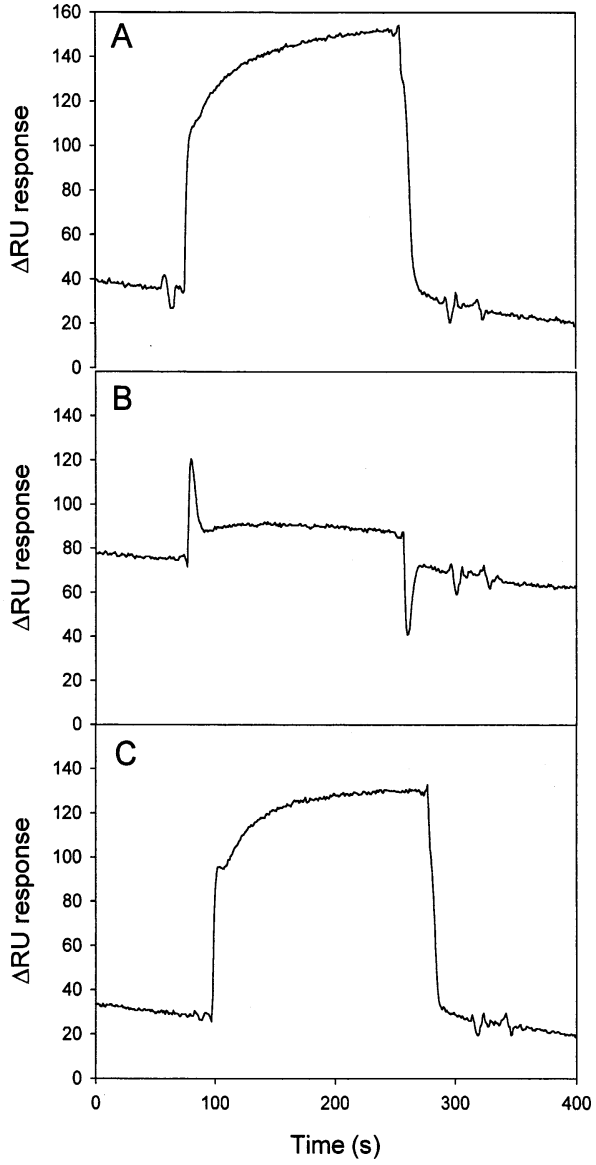


**Figure 4 Sensorgrams showing the SPR response to the reversible binding of L-Phe, D-Phe and 3-(2-thienyl)-L-alanine to tetrameric wt-hPAH**

Sensorgram (**A**) shows the response to binding of L-Phe (2 mM), (**B**) the binding of D-Phe (2 mM) and (**C**) the binding of 3-(2-thienyl)-L-alanine (2 mM) to tetrameric wt-hPAH. Immobilization of wt-hPAH resulted in a ΔRU of 25500 RU, consistent with 0.122 pmol enzyme/mm$^2$ (0.49 pmol subunit/mm$^2$). Calculations are described in the Materials and methods section. ΔN(1–102)/ΔC(428–452)-hPAH was immobilized to the chip of the reference cell ($\sim$ 0.4 pmol subunit/mm$^2$).

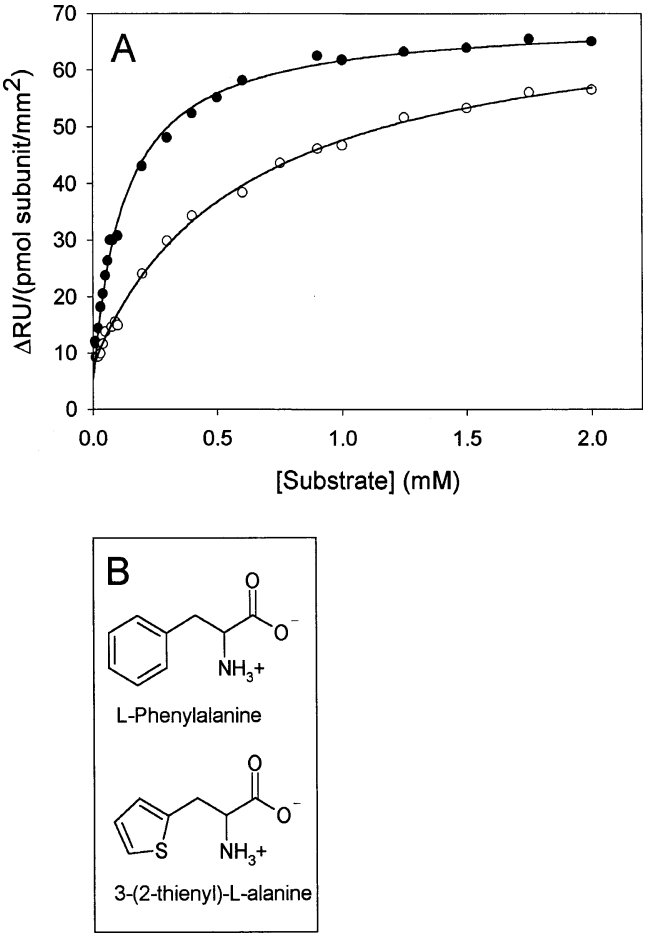


**Figure 5 Effect of 3-(2-thienyl)-L-alanine concentration on the SPR response isotherm with immobilized tetrameric wt-hPAH**

(**A**) Concentration-dependence for the SPR response induced by 3-(2-thienyl)-L-alanine (○) as compared with that by L-Phe (●). (**B**) Structure of L-Phe and 3-(2-thienyl)-L-alanine. The response isotherm for L-Phe represents the average of two separate titration experiments with a basal mean RU-value of 25090 for the immobilized wild-type enzyme, consistent with 0.12 pmol enzyme/mm$^2$ (0.48 pmol subunit/mm$^2$). Immobilization of wt-hPAH for the 3-(2-thienyl)-L-alanine assay resulted in a response of 25300 RU, consistent with 0.12 pmol enzyme/mm$^2$ (0.48 pmol subunit/mm$^2$). Calculations are described in the Materials and methods section. The truncated form ΔN(1–102)/ΔC(428–452)-hPAH was immobilized to the reference cell ($\sim$ 0.4 pmol subunit/mm$^2$).

suitable crystals of the full-length tetrameric form and its binary complex with L-Phe have not been available so far, the structural changes related to the substrate-induced internal motions are only partly understood.

The isolated tetrameric and dimeric wt-hPAH have stable chromatographic (at 4 °C) and distinctly different kinetic properties (assayed at 25 °C) [24], and dynamic light scattering [2] and SPR analyses [14] have excluded the contribution of a dissociation–association equilibrium as an explanation of the substrate-induced hysteresis effect (see below). In the present study, the maximum SPR response in the binding isotherm for L-Phe was almost the same for the dimeric wt-hPAH, the dimeric truncated form ΔC(428–452)-hPAH (containing the regulatory and catalytic domains) and tetrameric wt-hPAH (Figure 2), indicating that tetramerization is not a requirement for the SPR response and that the tetramerization domain does not contribute significantly to the global conformational change as measured by SPR. Thus, the SPR analyses support a model in which the conformational transition involves both the regulatory and catalytic domains, since the catalytically very active double truncated form ΔN(1–102)/ΔC(428–452)-hPAH shows no hysteresis and only a barely detectable non-time-dependent SPR response to L-Phe binding (Figures 1A and 1C). It should be noted further that, although the superposition of the crystal structures of the binary and ternary complexes of this truncated form has demonstrated structural changes in the catalytic domain when substrate is bound [30], these changes are not detected by SPR, but they are considered to trigger global conformational changes throughout the entire molecule (see [30] and below).

Whereas the intrinsic tryptophan fluorescence and UV difference spectroscopy (Table 1) primarily reflect changes in the micro-

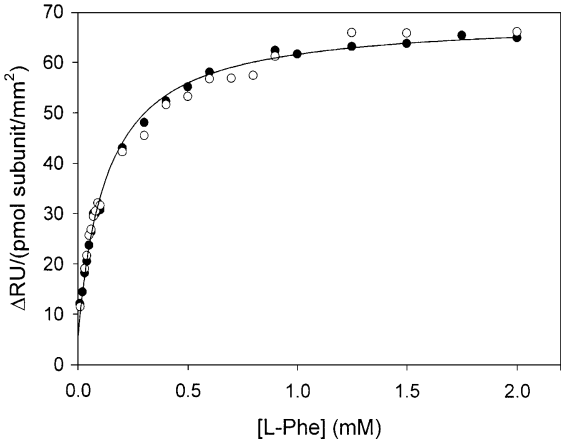


**Figure 6 Effect of phosphorylation on the L-Phe induced SPR response with immobilized tetrameric wt-hPAH**

Effect of L-Phe concentration on immobilized non-phosphorylated (●) and phosphorylated (○) wt-hPAH. The response isotherm for the non-phosporylated enzyme represents the average of two separate titration experiments with a basal mean RU-value of 25090 RU for the immobilized enzyme, consistent with 0.12 pmol enzyme/mm$^2$ (0.48 pmol subunit/mm$^2$). Immobilization of the phosphorylated form resulted in a basal RU-value of 26500 RU, consistent with 0.127 pmol enzyme/mm$^2$ (0.508 pmol subunit/mm$^2$). The truncated form ΔN(1–102)/ΔC(428–452)-hPAH was immobilized to the reference cell (~ 0.4 pmol subunit/mm$^2$). For calculations, see the Materials and methods section.

environment of specific aromatic amino acids in the individual subunits, the other manifestations of conformational changes, including the SPR response, are related to global conformational changes in the dimer/tetramer, i.e. motions which are likely to be intrinsically more complex.

### The dynamics of the reversible conformational transition

In general the hysteretic transition in an enzyme can result from a conformational change (isomerization), a dissociation–association reaction of the enzyme oligomeric structure, or a direct displacement of a bound ligand by a second ligand [31]. For the tetrameric form of PAH several independent criteria (Table 1) support the conclusion that a global conformational change represents the major contribution to the catalytic activation by its substrate. The SPR analyses in the present study have given additional information on the dynamics of this process. Thus both the on- and off-rates for the SPR response, related to the binding and dissociation of L-Phe, respectively, are clearly biphasic at temperatures < 25 °C (Figures 3C and 3D). At 10 °C the enzyme relaxes at two different rates, which is compatible with the presence of at least two major activation barriers for the conformational change in the transition from the 'T'- to the 'R'-state of the enzyme and vice versa. Since the crystal structure of the full-length tetrameric/dimeric enzyme is not yet available, the molecular basis of this biphasic transition is not known. However, the 3D structure of truncated forms support the conclusion that hinge-bending movements of subdomains are involved [27,28,30]. First, in the crystal structure analyses of the dimeric ΔC(428–452)-rPAH, two potentially flexible loops or hinge regions (i.e. residues Glu-31 to Ala-34, and Asp-111 to Val-117) have been proposed in the regulatory domain that may permit a motion of this domain relative to the catalytic domain, including an N-terminal inhibitory autoregulatory sequence (residues 19–29) [27,32]. Secondly, the recently solved crystal

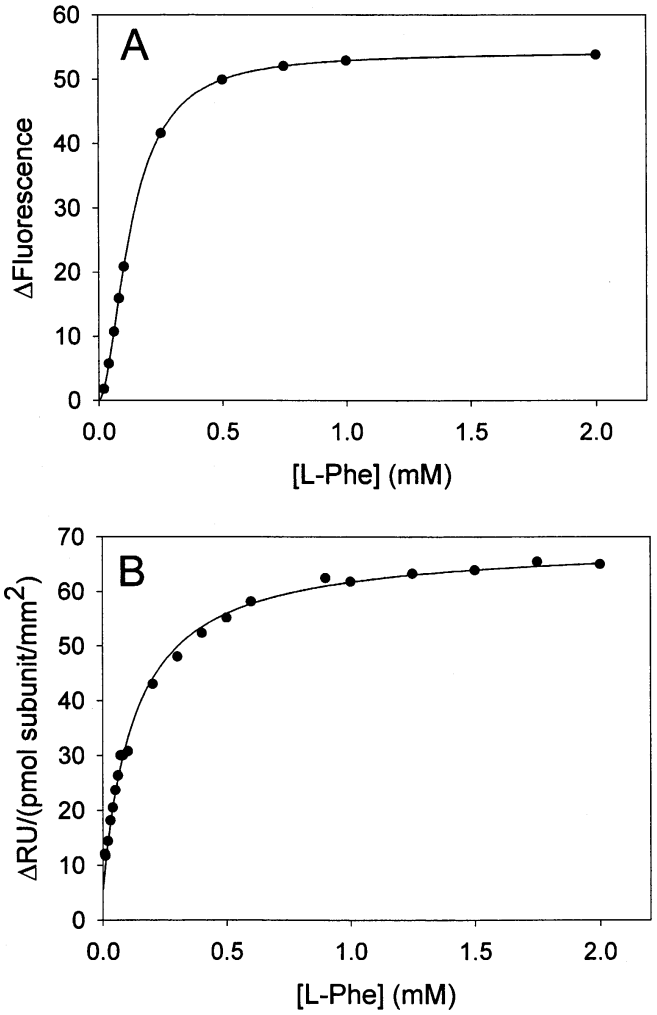


**Figure 7 Comparison of the steady-state equilibrium response on the binding of L-Phe to tetrameric wt-hPAH as determined by intrinsic tryptophan fluorescence and SPR analyses**

(**A**) Increase in fluorescence (ΔF) was measured as a function of L-Phe and D-Phe concentration, and the small, unspecific contribution that came from added D-Phe (change in the baseline as a result of added amino acid) was subtracted from the L-Phe signal. The titration was performed at 25 °C in a buffer containing 20 mM Hepes and 0.2 M NaCl, pH 7.0, and a concentration of the enzyme that gave an absorbance of 0.02 at 280 nm. Excitation and emission were at 295 nm and 358 nm, respectively. (**B**) SPR response isotherm for L-Phe represents the average of two separate titration experiments for tetrameric wt-hPAH with a mean basal ΔRU-value of 25090, consistent with 0.12 pmol immobilized protein/mm$^2$ (0.48 pmol subunit/mm$^2$). For calculations, see the Materials and methods section. The truncated form ΔN(1–102)/ΔC(428–452)-hPAH was immobilized to the reference cell (~ 0.4 pmol subunit/mm$^2$).

structures of the binary and ternary complexes of the truncated form ΔN(1–102)/ΔC(428–452)-hPAH [30,33] have revealed an intrasubunit hinge region (residues Phe-219–Phe-240), and that substrate binding to the binary complex hPAH-Fe(II)·$BH_4$ triggers a large-scale motion of a subdomain as well as additional structural movements throughout the molecule which adopts a slightly more closed conformation or compact structure [30].

Interestingly, a biphasic transition has also been observed for the thermal denaturation of tetrameric wt-hPAH, as measured by differential scanning calorimetry, which was interpreted to reflect a sequential unfolding of the regulatory domain (average $T_m$ ~ 46 °C) and the catalytic domain (average $T_m$ ~ 54 °C) [34].

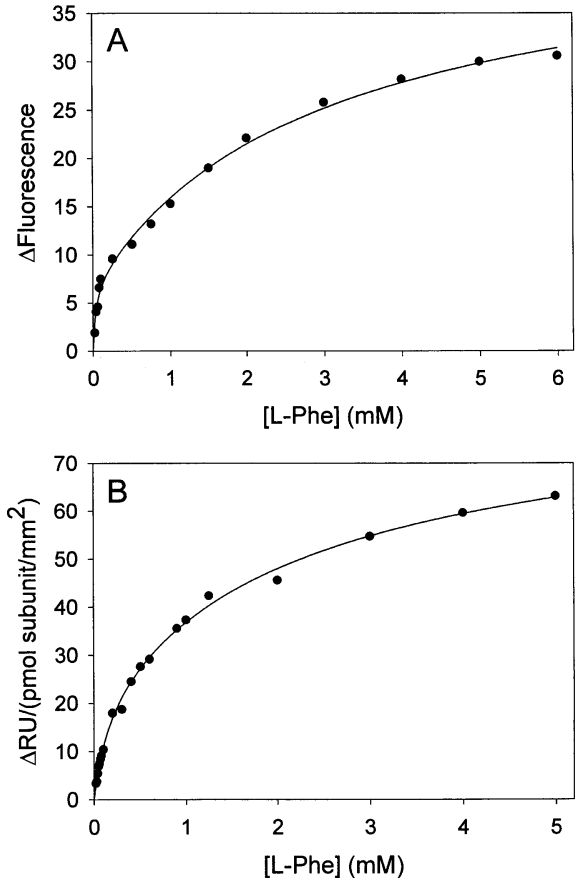


**Figure 8 Comparison of the steady-state equilibrium response on binding L-Phe to the truncated form ΔC(428–452)-hPAH as determined by intrinsic tryptophan fluorescence and SPR analyses**

(**A**) Change in fluorescence intensity (ΔF) was measured as a function of L-Phe and D-Phe concentrations, and the small, unspecific contribution that came from added D-Phe (change in base-line as a result of added amino acid) was subtracted from the L-Phe signal. The titration was performed at 25 °C in a buffer containing 20 mM Hepes and 0.2 M NaCl, pH 7.0, and a concentration of the enzyme that gave an absorbance of 0.02 at 280 nm. Excitation and emission were at 295 nm and 358 nm, respectively. (**B**) The effect of L-Phe concentration on the steady-state equilibrium SPR response of immobilized ΔC(428–452)-hPAH. Immobilization of ΔC(428-452)-hPAH resulted in a ΔRU-value of 14750 RU, consistent with 0.162 pmol enzyme/mm$^2$ (0.324 pmol subunit/mm$^2$). For calculations, see the Materials and methods section. The truncated form ΔN(1–102)/ΔC(428–452)-hPAH was immobilized to the reference cell ($\sim$ 0.3 pmol subunit/mm$^2$).

### The relationship between the global conformational transition and the co-operative binding of L-Phe

Tetrameric wt-hPAH, like other mammalian forms of PAH, binds L-Phe with positive co-operativity as determined by steady-state kinetics and equilibrium binding based on intrinsic tryptophan fluorescence measurements [24] (Tables 2 and 4). By contrast, the dimeric wt-hPAH and the dimeric truncated form ΔC(428–452)-hPAH revealed a hyperbolic dependence of L-Phe concentration in both kinetic and equilibrium binding studies (Figure 8 and Tables 2 and 4). When the L-Phe-induced global conformational transition was followed by SPR analyses, the tetrameric (Figure 1) and the dimeric form (Figure 2A) of wt-hPAH revealed almost the same SPR response both in terms of rate (end-point reached at $\sim$ 3 min) and maximum response at the end-point of the non-co-operative binding isotherm. The slightly different response isotherm of tetrameric hPAH measured by tryptophan fluorescence (Figure 7) can be explained by the basic difference between the two analytical methods. Thus, whereas the SPR method measures the global change in the refractive index of the protein [14], either as free dimer (Figure 2A) or present in a tetramer (dimer of dimers) (Figure 1), the change in the intrinsic tryptophan fluorescence reflects a change in the microenvironment of Trp-120 [12] in each subunit of the tetramer which becomes more exposed to the solvent on L-Phe binding [11,12], and with a co-operative type of binding isotherm for the wild-type tetramer only ([24] and Table 4). By contrast, on spectrophotometric titration (i.e. perturbation of both tyrosine and tryptophan residues) of rPAH the binding of L-Phe followed a hyperbolic saturation curve [11].

### On the L-Phe binding site related to the global conformational transition and catalytic activation

Our findings also contribute to resolve the debate [4,14] about the regulatory binding site for L-Phe in PAH. On the basis of equilibrium binding studies with labelled L-Phe, a regulatory binding site for L-Phe, distinct from the catalytic site, has been proposed [35,36]. Subsequently the putative regulatory site was suggested to be located somewhere in the regulatory domain, at the interface between the two subunits in the dimer [27,37], although no direct structural evidence has been presented so far for such a site. This view, therefore, has been challenged on the basis of 3D structural studies [30,38,39], all supporting the view that the regulatory and the catalytic active site are the same, and that the homotropic co-operative binding of L-Phe results from its binding at the active site [38]. First, $^1$H-NMR studies on L-Phe bound to wt-hPAH and its computer-based molecular docking into the crystal structure of the truncated dimeric form ΔC(428–452)-rPAH, containing the catalytic and N-terminal regulatory domain, strongly argue against the possibility of a second regulatory binding site distinct from the active site [39]. Secondly, the crystal structures of the binary and ternary complex of the double truncated form ΔN(1–102)/ΔC(428–452)-hPAH have revealed two different conformational states of the two structures. Thus, local conformational changes are induced at the active-site crevice structure by substrate binding [30], which is considered to trigger the global conformational transition of the full-length protein. Thirdly, the present study supports further a regulatory function of L-Phe bound at the active site. Thus the $[S]_{0.5}$-values (L-Phe) observed for the substrate-induced conformational transition in wt-hPAH tetramer ($97 \pm 6\ \mu$M), wt-hPAH dimer ($158 \pm 11\ \mu$M) and ΔC(428–452)-hPAH dimer ($756 \pm 64\ \mu$M) in the SPR analyses correlate well with the respective $[S]_{0.5}$-values obtained from the equilibrium binding of L-Phe, as determined by the change in intrinsic tryptophan fluorescence (i.e. $[S]_{0.5}$-values of $145 \pm 5\ \mu$M for wt-hPAH tetramer and $817 \pm 179\ \mu$M for ΔC(428–452)-hPAH dimer), and in steady-state kinetic analysis (i.e. $[S]_{0.5}$-values of $135 \pm 11\ \mu$M for the wild-type tetramer, $324 \pm 11\ \mu$M for the wild-type dimer and $845 \pm 169\ \mu$M for the C-terminal truncated dimeric form). Finally the substrate analogue 3-(2-thienyl)-L-alanine, which binds at the active site with the same orientation and specific interactions as L-Phe [30,39], triggers a time-dependent SPR response comparable with that observed with L-Phe (Figure 4 and Table 3). This finding was expected on the basis of enzyme kinetic studies [29] and the NMR binding studies, in which 3-(2-thienyl)-L-alanine was found to displace L-Phe from its specific binding at the active site [39].

### The effect of substrate analogues on the conformational transition

The substrate specificity of PAH has been extensively studied by Kaufman and Mason [29]. For maximum activity, a substrate

including an unmodified alanine must be attached to an aromatic ring, that may contain a number of substitutions and still be hydroxylated, as long as the alanine part is intact. Of particular interest was the finding that 3-(2-thienyl)-L-alanine is a substrate for PAH [29]. This analogue also triggers a time-dependent SPR response comparable with that observed with L-Phe (Figures 4 and 5). As expected from the substrate specificity studies [29] D-Phe was found to be unable to induce a SPR response similar to L-Phe at concentrations up to 2 mM (Figure 4B), indicating that the slow time-dependent conformational transition is stereospecific for L-Phe [and 3-(2-thienyl)-L-alanine].

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