Probing the Role of Crystallographically Defined/Predicted Hinge-bending Regions in the Substrate-induced Global Conformational Transition and Catalytic Activation of Human Phenylalanine Hydroxylase by Single-site Mutagenesis*

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Phenylalanine hydroxylase (PAH) is generally considered to undergo a large and reversible conformational transition upon 1-Phe binding, which is closely linked to the substrate-induced catalytic activation of this hysteric enzyme. Recently, several crystallographically solvent-exposed hinge-bending regions including residues 31–34, 111–117, 218–226, and 425–429 have been defined/predicted to be involved in the intra-protemer propagation of the substrate-triggered molecular motions generated at the active site. On this basis, single-site mutagenesis of key residues in these regions of the human PAH tetramer was performed in the present study, and their functional impact was measured by steady-state kinetics and the global conformational transition as assessed by surface plasmon resonance and intrinsic tryptophan fluorescence spectroscopy. A strong correlation (\( r^2 = 0.93–0.96 \)) was observed between the 1-Phe-induced global conformational transition and \( V_{\text{max}} \) values for wild-type human PAH and the mutant forms K113P, N223D, N426D, and N32D, in contrast to the substitution T427P, which resulted in a tetrameric form with no kinetic cooperativity. Furthermore, the flexible intra-domain linker region (residues 31–34) seems to be involved in a more local conformational change, and the biochemical/biophysical properties of the G33A/G33V mutant forms support a key function of this residue in the positioning of the autoregulatory sequence (residues 1–30) and thus in the regulation of the solvent and substrate access to the active site. The mutant forms revealed a variably reduced global conformational stability compared with wild-type human PAH, as measured by thermal denaturation and limited proteolysis.

Phenylalanine hydroxylase (PAH,\(^1\) phenylalanine 4-monoxygenase, EC 1.14.16.1) is a non-heme iron monooxygenase that catalyzes the hydroxylation of \( L-Phe \) to \( L-Tyr \) in the presence of the pterin cofactor (\( R^\prime \)-tetrahydro-5,6,7,8-tetrahydrobiopterin (\( BH_4 \)) and dioxygen, the rate-limiting step in \( L-Phe \) catabolism. Mutations in human PAH (hPAH), leading to altered kinetic properties and/or reduced in vivo stability of the enzyme, are associated with the autosomal recessive metabolic disorder phenylketonuria/hyperphenylalaninemia, and so far 439 putative disease-causing alleles have been identified in the PAH gene coding for a 452-residue polypeptide (\( 1 \)).

Mammalian PAH is a homotetramer (in equilibrium with a dimeric form) with the protomer consisting of three functional domains, i.e. a regulatory, a catalytic (core), and an oligomerization domain (for review, see Ref. 2). The tetramer is a dimer of two conformationally different dimers, denoted AC and BD, related by a 2-fold noncrystallographic symmetry axis (\( 3 \)). Based on a number of independent biochemical and biophysical criteria, it is generally accepted that the enzyme exists in at least two different activity and conformational states (4–8), and that it undergoes a large scale but relatively slow (seconds-to-minutes) global conformational transition (isomerization) upon binding of its substrate \( L-Phe \), which is characteristic of a hysteretic enzyme (5–8). Thus, the catalytic activation of tetrameric hPAH by its substrate can be represented by the equilibrium in Equation 1 (6, 8),

\[
PAH + Phe \rightleftharpoons PAH^\star Phe
\]

where PAH\(^\star\)-Phe represents the binary complex of the relaxed high activity state of the primed enzyme (PAH-Phe), and \( k_2 \) represents the slow (hysteretic) conformational transition. Thus, the binding of the substrate (7), as well as the pterin cofactor (9, 10), occurs by an induced-fit mechanism, with a perturbation of the structure of both the enzyme protomer and the ligand. Although the specific regions involved in the reversible conformational transition and catalytic activation of the tetramer, related to substrate binding at the active site, are only partly characterized (7, 11), the three-dimensional crystal structures of different truncated forms of hPAH (3, 12) and rat PAH (rPAH) (13) have given valuable insights into the molecular mechanism of substrate activation. Thus, hinge-bending regions in the catalytic domain have been identified to be involved in this isomerization on the basis of two conformational states (conformers) of the catalytic core structure (7, 11), and three additional inter- and intra-domain hinge-bending regions, schematically shown in Fig. 1, have been predicted from the structure of complementary truncated forms of hPAH (3, 14) and rPAH (13), for which only one x-ray conformer is yet

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\( ^1 \) The abbreviations used are: PAH, phenylalanine hydroxylase; BH\(_4\), \((\text{6R})\)-erythro-5,6,7,8-tetrahydrobiopterin; hPAH, human phenylalanine hydroxylase; PKU, phenylketonuria; rPAH, rat phenylalanine hydroxylase; SPR, surface plasmon resonance; wt, wild-type; RU, resonance units.
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available. There is, however, no crystal structure for any full-length tetrameric/dimeric PAH, which leaves unsolved the overall mechanism of the intramolecular propagation of the conformational changes initiated by the cooperative binding of L-Phe at the active site (11).

In order to gain further insight into the role of the crystallographically defined/predicted hinge-bending regions in the substrate-induced conformational transition and the related catalytic activation, single amino acid substitutions were made at key residues in full-length hPAH as follows: (i) in the flexible intradomain linker region (residues 31–34) of the regulatory domain structure that follows the N-terminal autoregulatory sequence (13); (ii) in the regulatory-to-catalytic inter-domain loop structure (residues 111–117) (13); (iii) in an intradomain hinge-loop region (residues 218–226) of the catalytic core domain structure (11); and (iv) in the dimerization-to-tetramerization loop structure (residues 425–429) (3) preceding the C-terminal amphipathic α-helix involved in tetramerization by three-dimensional domain swapping (Figs. 1 and 2). A key objective of the present paper was to study the effects of the structural perturbations of hinge flexibility, resulting from specific mutations, on the catalytic and global conformational properties of the tetrameric form of the enzyme. Intrinsic tryptophan fluorescence spectroscopy and surface plasmon resonance (SPR) were used to follow in real time the slow (hysterical) global conformational transition (k2 in Equation 1) that is triggered by the reversible binding of L-Phe to hPAH (2, 6, 8).

EXPERIMENTAL PROCEDURES

Materials—The primers for site-directed mutagenesis were obtained from MWG Biotech AG (Ebersberg, Germany). The QuikChange™ site-directed mutagenesis kit was from Stratagene. The Big Dye™ terminators ready reaction mix used to prepare DNA for sequencing was delivered by Applied Biosystems, and the DNA sequencing was carried out on an ABI Prism™ 377 DNA sequencer (Applied Biosystems). The restriction proteases factor Xa and enterokinase were obtained from Protein Engineering Technology ApS (Aarhus, Denmark) and Invitrogen, respectively. The reagents and the sensor chip CM5 used in the SPR analyses were purchased from Biacore AB (Uppsala, Sweden). The (6R)-erythro-5,6,7,8-Tetrahydrobiopterin was from the laboratory of Dr. B. Schircks (Jona, Switzerland). Trypsin and trypsin inhibitor were obtained from Sigma.

Site-specific Mutagenesis—The specific mutations addressed in the present study were selected based on predictive protein mutation stability calculations obtained by submitting the full sequence to the program K13P. When these calculations predicted that a substitution (mutated nucleotides are shown in boldface type): GCTTTCACGAGAT boldface type): GCTTTCACGAGAT (17).

Expression and Purification of hPAH—The wild-type and mutant fusion proteins were expressed in Escherichia coli with maltose-binding protein as the fusion partner and harvested after 2 h of induction with isopropylthio-galactoside, and the tetrameric fusion proteins were purified to homogeneity as described previously (17). The fusion proteins were cleaved by the restriction protease factor Xa or enterokinase, followed by size-exclusion chromatography at 4 °C (17) using a HiLoad Superdex 200 HR column (1.6 × 60 cm), prepacked from Amersham Biosciences. The isolated hPAH tetramers were collected, and the concentration was measured spectrophotometrically by using the absorption coefficient of the tetrameric form of hPAH. Wavelength scans were taken between 200 and 600 nm by using a 1-cm quartz cells, and the extinction coefficients at the peak maxima (Apeak) were calculated as the average of four scans; buffer scans were subtracted from the protein spectra. The global conformational stability, as measured by thermal denaturation (range 25–70 °C), was monitored by following the changes in the ellipticity at 222 nm at a constant heating rate of 1 °C/min. The apparent melting temperature (Tm) values were determined from the temperature at which 50% of the protein (Tm = 1/2 ln(2)) was unfolded by using the standard analysis program provided with the instrument. The first derivative profile was further resolved (deconvoluted) into individual overlapping Tm transitions, using a Gaussian distribution function in the PeakFit software program (SPSS Inc., Chicago).
Structural Studies—The crystallographically determined three-dimensional domain structure and hinge-loop regions of the truncated forms ΔN102-hPAH (lacking most of the N-terminal regulatory domain), ΔN102/ΔC24-hPAH, and ΔC24-hPAH (lacking the C-terminal tetramerization motif) were based on the Protein Data Bank files 2PAH (3), 1PHZ (12), 1J8T/1J8U (10), 1KWQ (7), 1MKM/1MMT (11), and 1PHZ (13). Fig. 2 was prepared using the structures 2PAH (3) and 1PHZ (13) and the programs InsightII and DS ViewerPro (Accelrys). The molecular motions of the catalytic domain of hPAH on substrate binding can be observed at (molmovdb.mbb.yale.edu/cgi-bin/morph.cgi?ID = 571404–31794) (11).

RESULTS

Rationale for Choosing Specific Residues for Mutagenesis—The schematic presentation of the domain structure of the wt-hPAH protomer shown in Fig. 1 also highlights the inter-domain and intra-domain solvent-exposed hinge-loop regions where single-site mutations were introduced. First, the predicted mutagenesis. Lys113 was substituted with a proline, as found in substrate binding (13). On the basis of the prediction of protein represent another center of hinge-bending motion as a result of secondary structure, connecting the regulatory domain with the catalytic domain, in rPAH has been proposed to represent another center of hinge-bending motion as a result of substrate binding (13). On the basis of the prediction of protein mutation stability changes, residue 113 was selected for mutation (14). For comparison, the functional effects of the N32D mutation were also studied.

Expression and Isolation of Wild-type hPAH and Its Mutant Forms—Fusion proteins of wt-hPAH and its mutant forms N223D, K113P, N426D, T427P, N32D, and G33A/G33V were all expressed in high yields in the TB1 strain of E. coli. The cells were harvested after 2 h of induction with isopropylthio-β-D-galactoside at 28 °C, i.e. conditions that almost completely prevent the deamidation of three labile Asn residues (at positions 32, 133, and 376), which occurs on longer induction times and higher temperatures (15, 18, 21) and results in a partly activated wild-type enzyme (characterized by a higher catalytic efficiency, a higher Hill coefficient, and an increased affinity toward L-Phe). Cleavage of the fusion proteins and subsequent isolation of their oligomeric forms by size-exclusion chromatography resulted in a variable recovery of soluble tetrameric forms (data not shown), but for all mutants recovery was in amounts sufficient for the present biochemical and biophysical studies.

Limited Proteolysis and Thermal Stability—Limited tryptic proteolysis is a sensitive probe for conformational changes in hPAH induced by non-enzymatic deamidation of labile asparagine residues (18), phosphorylation of Ser16 (22), ligand binding (23), as well as in several disease (24) and non-disease (14, 15) related mutant forms. In the present study 78% of the wild-type enzyme was found to be uncleaved after 30 min at the selected experimental conditions in the absence of L-Phe (Table 1). Some of the mutant enzymes revealed an increased susceptibility to limited proteolysis, with a full-length recovery of 67% for N223D and 74% for G33A after 30 min, whereas only 40% of T427P was found to be undigested (Table 1). A similar extent of cleavage as wt-hPAH was found for the N426D mutant, whereas the N32D mutant was slightly more resistant to proteolytic degradation, with a full-length recovery of 84% after 30 min (Table 1). The hinge-bending region (residues 111–117), representing the linker region between the regulatory and catalytic domains, contains three Lys residues in sequence and is one of the sensitive cleavage sites on limited proteolysis with trypsin. Thus, as expected, the K113P mutant form was more resistant to limited proteolysis than the wild-type, with 88% uncleaved protein after 30 min of proteolysis (Table 1), and the disparity was even more pronounced in the presence of substrate (1 mM L-Phe) with 68% full-length enzyme observed for the K113P mutant compared with 29% full-length wt-hPAH (data not shown).

All the mutant forms revealed an ordered secondary structure similar to the wt-hPAH, as assessed by far-UV CD spectroscopy (range 200–260 nm), and no significant change in the CD spectra (notably in the apparent α-helix content) was observed in the presence of L-Phe (data not shown). In the thermal denaturation studies two partially overlapping transitions were observed for the wild-type tetramer, with apparent T_m values at −46 and −54 °C (Fig. 3), which in the presence of L-Phe shifted to −54 and −59 °C, respectively. In general, the
Conformational stability of the tetrameric wild-type and mutant forms of hPAH as assessed by CD-monitored thermal denaturation and limited proteolysis with trypsin

| hPAH tetramer     | Thermal denaturation followed by CD | Limited proteolysis with trypsin<sup>a</sup> | Full-length hPAH |
|-------------------|-----------------------------------|---------------------------------------------|------------------|
|                   | 1st transition                    | 2nd transition                              |                  |
|                   | °C                                | °C                                          | %                |
| Wild-type<sup>b</sup> | 45.5 ± 0.8                        | 53.5 ± 0.2                                  | 77.7             |
| N223D             | 42.6 ± 0.7                        | 52.0 ± 0.9                                  | 66.9             |
| K113P             | 42.1 ± 0.1                        | 51.2 ± 0.4                                  | 88.0             |
| N426D             | 46.4 ± 0.5                        | 53.9 ± 1.0                                  | 78.3             |
| T427P<sup>a</sup>  | 40.7 ± 0.2                        | 50.2 ± 0.4                                  | 40.2             |
| G53A              | 42.2 ± 1.4                        | 51.9 ± 0.1                                  | 74.1             |
| N32D              | 46.8 ± 0.5                        | 54.6 ± 0.3                                  | 84.0             |

<sup>a</sup> Values correspond to percentage of full-length hPAH remaining after 30 min of a proteolysis reaction at 25 °C and at a hPAH to trypsin ratio of 200:1 (by mass).
<sup>b</sup> In the presence of 1 mM l-Phe, the apparent thermal transitions shifted to higher temperatures, i.e. −54 and −59 °C for the wild-type and −45 °C for the T427P mutant.

The mutant forms G33A/G33V and K113P were all characterized by an increased basal activity and a reduced enhancement, i.e. 1.4-, 1.5-, and 2.2-fold, respectively, on preincubation with l-Phe (Table II and Fig. 4). Moreover, the K113P mutant tetramer was found to have a slightly increased affinity toward l-Phe ([$S_{\text{h}}$]₀.₅ = 218 ± 4 µM) and a 2-fold increase in the catalytic efficiency (Table II), mainly because of a large increase in $V_{\text{max}}$. The two other mutant forms, G33A/G33V, revealed slightly different kinetic properties (Table II). The largest change was observed for the G33V mutant with a decrease in the [$S_{\text{h}}$]₀.₅ value for l-Phe (N223D and T427P) (Table II).

A positive cooperativity of l-Phe binding is a characteristic feature of the wt-hPAH tetramer ($n_H = 1.5 ± 0.1$). Although a slightly lower Hill coefficient was observed for the mutant form revealed a positive cooperativity of l-Phe binding ($n_H = 1.5 ± 0.1$) with a [$S_{\text{h}}$]₀.₅ value of 238 ± 18 µM and a 4.9-fold activation by preincubation with the substrate at 25 °C (Table II and Fig. 4). The selected mutations were expected to alter one or more kinetic parameters, which was indeed observed (Table II and Fig. 4).

The tetrramer of the four mutant forms N223D, N426D, T427P, and N32D revealed a low basal catalytic activity, similar to wt-hPAH (Fig. 4). However, whereas the N426D and N32D mutants gave a close to normal substrate activation (5.1- and 4.4-fold, respectively), the N223D and T427P mutant tetramers were activated only 2.7- and 1.2-fold, respectively, on preincubation with l-Phe (Fig. 4 and Table II). Except for the N223D where a higher catalytic efficiency was observed, the other mutant forms were characterized by a reduced catalytic efficiency either as a result of a reduced $V_{\text{max}}$ effect (N223D) or an increase in the [$S_{\text{h}}$]₀.₅ value for l-Phe (N246D and T427P) (Table II).
tetrarmers G33A (n_H = 1.3 ± 0.1), G33V (n_H = 1.4 ± 0.1), N223D (n_H = 1.2 ± 0.1), and N426D (n_H = 1.4 ± 0.1), the K113P and N32D mutants revealed an increased positive cooperativity (n_H = 2.1 ± 0.1 and 2.2 ± 0.1, respectively) (Table II). By contrast, the T427P mutant tetramer revealed a noncooperative binding of L-Phe (i.e. n_H = 1.0 ± 0.1) (Table II). Most of the mutants revealed a slightly decreased affinity for the pterin cofactor BH₄ as compared with the wild-type tetramer, with the most dramatic decrease observed for the K113P mutant form (Table II).

Surface Plasmon Resonance and Intrinsic Tryptophan Fluorescence Measurements—SPR spectroscopy has been used successfully to monitor in real time the reversible and large scale global conformational transition that occurred in immobilized wt-hPAH upon L-Phe binding at the active site (6, 8) and was positively correlated to the change in intrinsic tryptophan fluorescence in solution (8). The time-dependent increase (within 3 min) in the SPR response to L-Phe injection obtained for the full-length wt-hPAH tetramer (6, 8) was also observed for all the mutant forms (Fig. 5C) with saturation reached at about 2 mM substrate, although the maximum equilibrium SPR response (ΔRU_max) in the binding isotherms varied (Fig. 5, A and C). For the N223D mutant ΔRU_max of −60% of the value observed for the wild-type was obtained. By contrast, a significant increase in the ΔRU_max value (−130%) was observed for the K113P tetramer (Fig. 5C). The N426D mutant displayed a similar binding isotherm as the wild type, whereas the T427P mutant revealed an −50% decrease in the ΔRU_max. The N-terminal mutants G33A and G33V also revealed a reduced ΔRU_max response, i.e. −30 and −50%, respectively, of wt-hPAH.

| hPAH tetramer | V_max | [S]₀.₅ | K_m | Hill coefficient | Fold activation | BH₄ | V_max | K_m |
|---------------|-------|--------|-----|-----------------|----------------|-----|-------|-----|
| Wild type     | 3484 ± 162 | 238 ± 18 | 0.72 | 1.5 ± 0.1 | 4.9 | 5325 ± 137 | 31 ± 3 |
| N223D        | 1984 ± 82  | 242 ± 42 | 0.41 | 1.2 ± 0.1 | 2.7 | 2342 ± 57  | 37 ± 2 |
| K113P        | 6192 ± 105 | 218 ± 4  | 1.40 | 2.1 ± 0.1 | 2.2 | 8358 ± 289 | 47 ± 3 |
| N426D        | 3672 ± 359 | 424 ± 20 | 0.44 | 1.4 ± 0.2 | 51  | 4396 ± 168 | 30 ± 4 |
| T427P        | 3168 ± 181 | 549 ± 31 | 0.29 | 1.0 ± 0.1 | 1.2 | 2842 ± 57  | 30 ± 1 |
| G33A         | 4366 ± 172 | 230 ± 20 | 0.97 | 1.3 ± 0.1 | 1.4 | 4727 ± 195 | 35 ± 3 |
| G33V         | 5163 ± 419 | 176 ± 28 | 1.50 | 1.4 ± 0.1 | 1.5 | 6411 ± 195 | 40 ± 2 |
| N32D         | 3736 ± 47  | 159 ± 3  | 1.20 | 2.2 ± 0.1 | 4.4 | 2752 ± 133 | 34 ± 2 |

Table II
Steady-state kinetic parameters of isolated wt-hPAH and its mutant form

Fig. 3. CD-monitored thermal denaturation of wild-type and mutant forms of full-length tetrameric hPAH. Top panel, the thermal denaturation profiles (range 25–70 °C) of wt-hPAH and the T427P mutant form were monitored by the change in the molar ellipticity at 222 nm at a constant heating rate of 1 °C/min, in the absence (solid line) and presence (dotted line) of 1 mM L-Phe. The apparent melting temperature (T_m) values were determined from the first derivative of the denaturation curve (inset). Bottom panel, the first derivative of the thermal denaturation curves for some of the hinge-related mutant forms (solid line) with the wt-hPAH as a reference (dashed line).
in contrast to N32D, which gave a saturating equilibrium binding response similar to the wild type (Fig. 5C). Moreover, the substrate concentration yielding half-maximum SPR response (\\(S_0.5\\)) in the binding isotherms was higher for some of the mutant forms (i.e. 162 ± 3 \( \mu \)M for N223D, 189 ± 7 \( \mu \)M for N426D, 282 ± 10 \( \mu \)M for G33A, and 381 ± 14 \( \mu \)M for G33V) as compared with the wild-type (136 ± 2 \( \mu \)M) (Table III). K113P demonstrated a slightly higher affinity for L-Phe (\\(S_0.5 = 120 ± 3 \mu M)\), whereas for T427P the affinity was the same as for the wild type, i.e. 135 ± 6 \( \mu \)M (Table III).

The tryptophan fluorescence spectrum of tetrameric wt-hPAH demonstrated an ~10 nm red shift and an increased quantum yield when binding L-Phe at a saturating concentration (20). This response was primarily related to a change in the microenvironment of Trp^{120} in each protomer, i.e. from a buried position in the ligand-free enzyme to a more solvent-exposed position in the substrate-activated form (20), and the tryptophan fluorescence has been used as a valuable conformational probe for both the wild-type and various truncated forms of the enzyme (8). Fig. 5B demonstrates a typical binding isotherm for L-Phe to wt-hPAH tetramer with a \(S_0.5\) value of 78 ± 2 \( \mu \)M and a Hill coefficient of 2.7 ± 2. For the wild type and four of the mutant tetramers (N223D, K113P, N426D, and N32D) a close positive correlation (\(r^2 = 0.98\), figure not shown) was observed between the \(\Delta F_{max}\) value at 358 nm (Fig. 5D) and the \(\Delta RU_{max}\) obtained by SPR (Fig. 5C) (see also below). However, the tryptophan fluorescence of the T427P mutation did not change significantly upon the addition of L-Phe. In general, a decrease in the Hill coefficient determined from the binding isotherms was observed for most of the mutant forms (Table III).

**The Relationship between the Substrate-triggered Large Scale Global Conformational Transition and Catalytic Activity**—Recent x-ray crystallographic analyses of the catalytic core domain structure of hPAH (7, 11) and spectroscopic (magnetic circular dichroism and x-ray absorption spectroscopy) studies on wt-rPAH and two disease-associated mutant forms (25) have provided valuable insights into the relation between structure and function of this enzyme. In both studies it was shown that substrate binding results in a change in the binding geometry/coordination of the catalytic iron and a reorientation of residues lining the active site, which represent major contributing factors to catalysis, and new insights into the catalytic mechanism were obtained. Moreover, two distinct conformational states (conformers) were observed in the crystal structure of the catalytic core domain representing the unliganded and substrate analog-bound forms of the enzyme (7, 11). This supports the original proposal by Shiman and Gray (5) that protein dynamics play a vital role in the PAH catalytic pathway. In the present study the biochemical/biophysical properties observed for the described mutant forms of full-length tetrameric hPAH have given additional information on the relationship between enzyme dynamics/flexibility and catalytic activity (\(V_{max}\\)). The catalytic activity of PAH was measured either by varying the L-Phe concentration or the BH_{4} concentration. Whereas varying substrate concentration resulted in sigmoid steady-state kinetics, the BH_{4} dependency demonstrated classical Michaelis-Menten kinetics, and a higher \(V_{max}\\) was also obtained for the latter (Table II). Thus, from Fig. 6 it is seen that the change in catalytic activity for both the L-Phe (Fig. 6A) and the BH_{4} (Fig. 6B) concentration curves upon mutation of defined/predicted hinge-bending residues in hPAH correlates strongly with the change in the substrate-induced global conformational transition (linear correlation coefficient \(r^2 = 0.93–0.96\) for N32D, K113P, N223D, N426D, and wt-hPAH tetramers, as measured by SPR and intrinsic tryptophan fluorescence spectroscopy. By contrast, G33A/G33V and T427P did not fit into this correlation (Fig. 6).

Although SPR and intrinsic tryptophan fluorescence detect different aspects of the L-Phe-triggered conformational change of the proteomer, the \(\Delta RU\\) and \(\Delta F\\) responses have complementary time windows and binding isotherms (8). The conformational transition observed by SPR (as a positive \(\Delta RU\\) value) is compatible with an increase in the hydrodynamic radius as measured by size-exclusion chromatography and dynamic light scattering (26, 27). The biphasic increase in refractive index (8) also defines the time scale for the conformational change (i.e. molecular motions) in this hysteretic enzyme. However, the exact molecular basis for the SPR response requires further biophysical studies, including volumetric techniques that may give further insights into hydration and intramolecular packing related to the transition.

**DISCUSSION**

The tetrameric form of mammalian PAH displays homotropic cooperative binding of the substrate L-Phe, as observed by steady-state enzyme kinetics and equilibrium binding studies (2, 8, 28, 29). The cooperativity has so far primarily been interpreted within the framework of the classical concerted two-state model of Monod et al. (30), i.e. as a reversible transition between a low affinity/low activity “T-state” and a high affinity/high activity “R-state” of the enzyme tetramer (5, 8, 26–28). However, the molecular basis for the cooperativity of L-Phe binding and its catalytic activation of the tetramer is only partly understood.

**Molecular Motions in the Catalytic Domain Triggered by Substrate Binding**—The crystal structures of two different conformational states have been obtained recently for the unliganded and the ternary complex of the dimeric double-truncated form of hPAH, with the substrate analogs 3-(2-thienyl)-L-alanine and L-norleucine (7, 11). The structures revealed that binding of substrate at the active site triggered large scale structural changes in the catalytic domain, including the active site crevice structure, representing the “epicenter” of the global conformational transition related to L-Phe activation observed in the full-length protomer and its related oligomeric forms (7, 11). The most prominent molecular motions in the structural transition were observed in the loop region comprising residues 134–139, flanked by two short \(\beta\)-helices (residues 125–133 and 140–142), with a maximum backbone displacement of 9.6 Å for Tyr^{130} (7, 11). In addition, two hinge-bending regions
centered at Leu<sup>197</sup> (in a kinked α-helix) and Asn<sup>223</sup> (in the 218–226 loop structure) were defined and found to act in concert upon substrate binding to create a large scale rigid-body movement that displaces the β-strands 333–336, 339–342, 363–366, and 385–389 as well as the α-helices 351–356, 369–372, and 392–403 (7, 11). By the substitution of N223D, this hinge region is likely to destabilize the structure by disrupting an H-bond to Gly<sup>218</sup> (Fig. 2A), resulting in an ~50% reduction in the measured global conformational change triggered by L-Phe binding (Fig. 5, C and D). Thus, in this mutant form the affinity for the substrate is not altered (Table II), but the basal catalytic activity and the fold activation by L-Phe preincubation are both significantly reduced (Fig. 4 and Table II). The results support the involvement of the 218–226 hinge region in the substrate-induced molecular motions in the catalytic domain as shown in the crystallographic studies (7, 11). Since no three-dimensional structure of the full-length tetrameric enzyme has yet been obtained, how the conformational changes observed in the catalytic domain are propagated to the N- and C-terminal domains of the protomer and affect the interactions between the subunits in the dimer/tetramer is an open question. Moreover, the molecular dynamics simulations of full-length hPAH have so far not been able to elucidate further the subset of motions and the dynamic pathway of the substrate-induced conformational changes (31). The major motions in this hysteresis, enzyme occur on longer time scales, i.e. seconds-to-minutes (2, 5, 6, 8), than are normally covered (nanoseconds) by the molecular dynamics simulations. Here we addressed this issue by studying the functional effects of site-directed mutagenesis of specific residues at the defined (11) and predicted (3, 13, 14) hinge-bending regions by spectroscopic methods, which allow the global conformational change to be followed in real time (6, 8).

**The Regulatory-to-Catalytic Interdomain Hinge-bending Region**—The functional properties observed here for the K113P mutant tetramer (with the wild-type sequence 111<sup>R</sup>DKKEKT<sup>177</sup> in rPAH, connecting the regulatory domain (residues 2–117) with the catalytic domain (residues 118–410), represents a center of interdomain movement (13). The Pro substitution will cause not only a change in the backbone conformation but also a loss of two H-bonds to the carboxyl groups of Asp<sup>27</sup> in the autoregulatory sequence and Asp<sup>145</sup> in the catalytic domain (Fig. 2A). Although both aspartates also form H-bonds to Arg<sup>252</sup> in the catalytic domain (Fig. 2A), the position of the autoregulatory
sequence in relation to the active site crevice structure may still be affected by the mutation through a weakening of the interactions between the regulatory and catalytic domains, and thus facilitate inter-domain movements upon l-Phe binding. The time-dependent increase in the refractive index of immobilized tetrameric and dimeric wt-hPAH triggered by l-Phe binding has been related to a global hysteretic conformational transition (isomerization), involving both the catalytic and the regulatory domains (8). The K113P mutation per se results in a repositioning (i.e. conformational change) of the flexible N-terminal autoregulatory sequence, as indicated by the 3.5-fold increase in the basal catalytic activity and a corresponding reduced catalytic activation on preincubation with l-Phe, indicating a more substrate-accessible active site. Thus, the 1.3-fold increase in the $\Delta RU_{\text{max}}$ response observed here for the K113P tetramer is compatible with a higher mobility of the regulatory domain (relative to the catalytic domain) being responsible for the overall increase in the refractive index of the immobilized enzyme on l-Phe binding.

**Hinge-bending Region in the Regulatory Domain That Controls Solvent and Substrate Access to the Active Site**—Structural and biochemical evidence has been presented in support of the proposal that a conformational flexibility of the N-terminal autoregulatory sequence (residues 1–30) is involved in the regulation of solvent and substrate access to the active site (13, 32–35). In the unliganded structure of a dimeric rPAH lacking the C-terminal 24-residue tetramerization motif (ΔC24-rPAH), a segment (residues 19–29) of the autoregulatory sequence extends over the active site crevice in the catalytic core domain, leaving only a relatively narrow access for the substrate (13). A rotation of the regulatory domain (relative to the catalytic domain) at the linker region $^{111}$RDKEKNT$^{117}$, triggered by l-Phe binding (discussed above), was considered to result in a movement of the autoregulatory sequence away from the active site including a hinge-bending motion centered at Gly$^{33}$ (13, 32). The biochemical and biophysical results obtained in the present study for the G33A/G33V mutant gives further support for this conclusion. Gly$^{33}$ is a buried residue (Fig. 2A), and its substitution by more bulky hydrophobic amino acids, such as Ala and Val, may introduce a restriction in the conformational space and movement at the hinge. Thus, both mutant forms revealed a markedly increased basal catalytic activity (3.2-fold for both) and a correspondingly reduced catalytic activation on preincubation with l-Phe (Fig. 4 and Table II), compatible with a more open active site crevice structure in the ligand-free mutant forms, probably because of a more permanent reorientation of the N-terminal arm at this hinge. Although the G33A/G33V mutations had comparable $V_{\text{max}}$ values to the wild type, they showed a reduced tryptophan fluorescence response and a reduced SPR response ($\Delta RU_{\text{max}}$) by $\sim$30% (G33A) and $\sim$50% (G33V) of the wild-type at 2 mM l-Phe (Fig. 5C) and thus did not fit into the linear activity-conformational change correlation observed for wt-hPAH and the other mutant forms (Fig. 6). The reduced conformational transition may be explained by a more restricted mobility of the N-terminal arm, which does not succeed in the full change of the local environment of the spatially closed Trp$^{126}$, and in the movements related to the N-terminal component of the SPR response. Most interesting, the effects of the mutations at Asn$^{32}$ and Gly$^{33}$ on the binding of l-Phe at the active site (Table II) support the proposal (31) that the overall conformation of the regulatory domain influences the cooperativity without directly binding the substrate, as compared with previous suggestions of a second regulatory binding site for l-Phe in this domain (5, 13, 27).

**The Dimerization-to-Tetramerization Interdomain Hinge-bending Region Mediating the Homotropic Cooperative Binding of l-Phe**—The crystal structure of an N-terminal truncated tetrameric hPAH (ΔN102-hPAH) has identified a five-residue hinge-bending region (L$^{205}$DNTQQ$^{209}$) in the dimerization-to-tetramerization inter-domain structure that links the two antiparallel $\beta$-strands (residues 411–415 and 420–424) with the 25-residue C-terminal amphiphatic $\alpha$-helix (residues 428–452), which forms an antiparallel coiled-coil structure by domain swapping in the tetramer (3). Substitution of Asn$^{206}$ with an Asp (as in the structurally related human tryptophan hydroxylase) and Thr$^{417}$ with a Pro (as in human tyrosine hydroxylase) resulted in mutant forms with quite different properties. The N426D tetramer revealed a $V_{\text{max}}$ and a Hill coefficient similar to that of wt-hPAH, but with a reduced catalytic efficiency (mainly a $[S]_{0.5}$ effect of l-Phe) (Table II). Moreover, it was activated 5-fold by l-Phe preincubation (Fig. 4 and Table II) and revealed a similar substrate-induced global conformational change as wt-hPAH (Fig. 5, C and D, and Fig. 6). By contrast, the T427P tetramer (as isolated) revealed almost no
activation (1.2-fold) by l-Phe, and its catalytic efficiency (Table II) was decreased to about 40% of wt-hPAH (mainly a $V_{\text{max}}$ effect). A decrease was also observed for the l-Phe-induced global conformational change as measured by SPR spectroscopy, whereas only a small decrease (negative response) in the intrinsic tryptophan fluorescence of Trp$^{129}$ was seen (Table III). It should be noted that the crystal structure of the ligand-free $\Delta N102$-hPAH tetramer shows a pronounced asymmetry (3), with two alternate conformations in the 425–429 hinge regions of the two adjacent dimers (AC and BD) in the tetramer, related by a 2-fold noncrystallographic symmetry axis (3). In both subunits of the dimer BD, Thr$^{427}$ in this loop establishes a stabilizing H-bond (3.2 Å) with the C-terminal carboxyl group of Lys$^{652}$ in the subunits of dimer AC (Fig. 2B), respectively. Most interesting, because of the asymmetry of the tetrameric structure, the 425–429 loop regions of dimer AC cannot interact with the C termini of dimer BD because the distance between the two residues is 5.4 Å. This implies that in the ligand-free wild-type tetramer two interactions will occur, one in each end of the coiled-coil motif, involving two monomers in different dimers (AC and BD). Moreover, molecular modeling has suggested that this hinge region also establishes several contacts with the regulatory domain of the same subunit (31) and thus may represent a possible pathway for the propagation of the substrate-induced global conformational changes initiated at the active site (the epicenter). By contrast, in tyrosine hydroxylase Thr$^{427}$ is substituted by a Pro residue, and the enzyme exists only as a symmetric tetramer in which the four subunits are related by a crystallographic 222 symmetry axis (36), and the enzyme shows no cooperativity of substrate binding. Thus, the T427P mutation in hPAH resulted in the loss of cooperativity, and instead of a stabilization of the tetrameric form, a shift in the tetramer ↔ dimer equilibrium in favor of free dimers (14) was observed, probably because of a loss of the asymmetry and the interactions between the subunits in the two dimers AC and BD (see above).

Global Conformational Stability of Wild-type and Mutant Forms of hPAH—None of the hinge-loop mutations studied here showed any significant change in the ordered secondary structure as assessed by their far-UV CD spectra, but minor conformational changes were suggested on the basis of the four complementary conformational probes used, including the relative thermal stability (Fig. 3). Previous thermal denaturation studies by differential scanning calorimetry and far-UV CD spectroscopy on the wild-type and truncated forms of hPAH (37) have suggested that the thermodynamically irreversible denaturation of the full-length wild-type tetramer occurs through a sequential unfolding of the N-terminal regulatory domains ($T_m \sim 46^\circ \text{C}$) and the catalytic domains ($T_m \sim 54^\circ \text{C}$) (37). Both transitions are shifted to higher temperatures in the presence of l-Phe (37) (Fig. 3). The observed effect of l-Phe binding on the biphasic thermal denaturation profile of wt-hPAH (37) (Fig. 3) further supports the interpretation of the SPR response in terms of a biphasic time course, possibly contributed by the two domains (8). Most of the tetrameric mutant forms revealed similar thermal denaturation profiles, with two resolved transitions (Fig. 3 and Table I). Moreover, the conformational changes induced by the mutations G33A, K113P, and N223D affected the $T_m$ value for the first transition (related to the regulatory domain), whereas the second transition was relatively unaffected. Most interesting, the thermal unfolding process of the T427P-hPAH tetramer presented essentially a single transition with a low $T_m$ value (41 °C). The T427P mutation at the 425–429 hinge may thus be responsible for a destabilization of both the regulatory and catalytic domains, which unfold at around the same $T_m$ value. Overall, there seems to be a good correlation between the extent of limited proteolysis and the thermal stability of the mutant enzymes when compared with the wild-type, probably related to differences in their intramolecular packing of the structural elements in the tetramers.

Concluding Remarks—Structural dynamics is essential for the biological function of proteins, and hinge-bending motions usually occur in proteins that have two or more domains connected by linkers (i.e. hinges/loops) and are frequently involved in ligand-induced protein conformational changes. PAH is such a protein in which the binding of substrate (L-Phe) at the active site initially triggers local conformational changes at this site (the epicenter), which are propagated globally through several hinge-bending motions, as partly demonstrated by molecular motion analyses of the catalytic core enzyme (7, 11; see also under "Experimental Procedures"). The present site-directed mutagenesis study provides further support for the structure-based model in which defined hinge-bending regions have been demonstrated (7, 11) or predicted (3, 13, 14) crystallographically to be involved in the substrate-induced global conformational transition. Most interesting, a strong correlation ($r^2 = 0.93–0.96$) was observed relating the catalytic activity of the wild-type and the N32D, K113P, N223D, and N426D mutant tetramers to the l-Phe-induced large scale global conformational changes as assessed by both SPR and intrinsic tryptophan fluorescence spectroscopy. This correlation supports previous conclusions, based on crystallographic data (7, 11), that substrate binds to the catalytic/active site where it triggers large scale structural changes in the catalytic domain, which are further transmitted to the dimer/tetramer and directly relate the global conformational change to the catalytic activation of the enzyme. The G33A/G33V and T427P mutant tetramers did not fit into this relationship, indicating a more restricted contribution of the related hinges to the global conformational transition, as measured by SPR and fluorescence spectroscopy. Further studies are required to uncover their exact involvement in the activation of PAH. In particular, three-dimensional structural studies of a full-length dimeric/tetrameric enzyme, in its ligand-free and substrate-bound form, are required to determine how the local conformational changes at the active site are propagated to the entire protomer as well as to the dimer/tetramer leading PAH from its "T-state" to its "R-state."

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