Domain structure and stability of human phenylalanine hydroxylase inferred from infrared spectroscopy

Rosana Chehin[1,a], Matthias Thorolfsson[b], Per M. Knappskog[c], Aurora Martinez[b], Torgeir Flatmark[b], Jose Luis R. Arrondo[a], Arturo Muga[a,*]

[a] *Department of Biochemistry and Molecular Biology, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain*
[b] *Department of Biochemistry and Molecular Biology, University of Bergen, Arstadveien 19, N-5009 Bergen, Norway*
[c] *Department of Medical Genetics, University of Bergen, Haukeland Hospital, N-5021 Bergen, Norway*

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Abstract We have studied the conformation and thermal stability of recombinant human phenylalanine hydroxylase (hPAH) and selected truncated forms, corresponding to distinct functional domains, by infrared spectroscopy. The secondary structure of wild-type hPAH was estimated to be 48% α-helix, 28% extended structures, 12% β-turns and 12% non-structured conformations. The catalytic C-terminal domain (residues 112–452) holds most of the regular secondary structure elements, whereas the regulatory N-terminal domain (residues 2–110) adopts mainly an extended and disordered, flexible conformation. Thermal stability studies of the enzyme forms indicate the existence of interactions between the two domains. Our results also demonstrate that the conformational events involved in the activation of hPAH by its substrate (L-Phe) are mainly related to changes in the tertiary/quaternary structure. The activating effect of phosphorylation, however, affects the secondary structure of the N-terminal domain of the protein.

Key words: Phenylalanine hydroxylase; Deletion mutation; Protein structure; Protein stability; Infrared

1. Introduction

Mammalian phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH, EC 1.14.16.1) is a tetrahydrobiopterin-dependent enzyme that catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine. PAH, a 452 residue protein, is found predominantly in liver and the absence or reduction in catalytic activity of the human enzyme leads to a serious disease (phenylketonuria). In this homotetrameric enzyme, the subunits contain an N-terminal regulatory domain, with a phosphorylation site (Ser-16), and a C-terminal domain with catalytic activity, which includes the tetramerization motif and whose steady-state kinetic and oligomeric properties have recently been characterized [1]. PAH is activated by preincubation with its substrate L-Phe, which seems to be an important mechanism for regulating the degradation of this essential amino acid in hepatocytes [2,3]. Phosphorylation/dephosphorylation of PAH at Ser-16 represents an additional post-translational regulatory modification of the enzyme, as studied in rat PAH [3] and human PAH (hPAH) [4,5]. Both regulatory mechanisms seem to be interrelated, suggesting the existence of interactions between the N- and C-terminal domains of the protein. Thus, L-Phe enhances the rate of phosphorylation of hPAH by the cAMP-dependent protein kinase (PKA) and the phosphorylated enzyme requires lower concentration of L-Phe for substrate activation [5].

The 3D structure of a truncated form (containing the catalytic and tetramerization domains) of the structurally and functionally related enzyme tyrosine hydroxylase (TH) has recently been determined [6]. Moreover, a dimeric truncated form of hPAH containing the catalytic domain has been crystallized [7] and its structure determined [8]. Although the 3D structure of these truncated enzyme forms provides an adequate frame to interpret the putative structural changes induced by substrate activation, they lack the regulatory N-terminal domain. In this context, spectroscopic methods, such as infrared (IR) spectroscopy allow to directly compare the conformational properties of both protein domains in solution. In the present study we have compared the secondary structure and stability of recombinant hPAH with its C-terminal and N-terminal domains, as well as measured the effects of activation by L-Phe and phosphorylation.

2. Materials and methods

2.1. Materials

DNA primers for PCR and sequencing were synthesized on an Applied Biosystems (ABI) model 392 synthesizer. *Taq* DNA polymerase was from Boehringer Mannheim. DNA sequencing was carried out by the Taq Dye DeoxyTerminator Cycle Sequencing Kit (ABI) using an automated DNA sequencer (model 373A from ABI). Factor Xa was obtained from New England Biolabs. Deuterium oxide was from Sigma. The catalytic subunit of the cAMP-dependent protein kinase (PKA) was purified from bovine heart as described [9].

2.2. Construction of vectors and site-directed mutagenesis

The wild-type (wt) cDNA of hPAH was subcloned into the pMAL-c2 vector (New England Biolabs), and the deletion mutant forms hPAH(Asp-112-Lys-452) (Δ1–111), corresponding to the catalytic domain, and hPAH(Ser-2-Gln-428) (Δ429–452), lacking the tetramerization motif, were prepared by PCR-based site-directed mutagenesis as described [1,10]. An N-terminal fragment, hPAH(Ser-2-Ser-110) (Δ111–452) corresponding to the N-terminal domain, was constructed by PCR mutagenesis. A premature stop signal in codon 111 was introduced in the hPAH cDNA (pMAL vector) using the mutagenic oligonucleotide (GTC CAT GAG CTT TCA *T*GA) and other primers previously described [10]. The mutant PCR fragment was subcloned into the *Xba*I/*Xho*I sites of the hPAH cDNA. Positive clones were sequenced to verify the mutagenesis and to exclude other mutations.

2.3. Expression and purification of recombinant hPAH forms

Expression of wt-hPAH, hPAH(Asp-112-Lys-452) and hPAH(Ser-

*Corresponding author. Fax: +34 (4) 4648500.
E-mail: gbpmuvia@lg.ehu.es

[1]Permanent address: INSIBIO, Department of Nutrition and Biochemistry, Chacabuco 461, Tucuman 4000, Argentina.

Abbreviations: hPAH, human phenylalanine hydroxylase; IR, infrared; MBP, maltose binding protein; TH, tyrosine hydroxylase; PKA, cyclic AMP-dependent protein kinase; wt-hPAH, wild-type human phenylalanine hydroxylase

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2-Gln-428) in *Escherichia coli* (TB1), purification of the fusion proteins by affinity chromatography on amylose resin followed by high-performance size exclusion chromatography, and cleavage by factor Xa was performed as described [1,4]. The tetrameric and dimeric forms of wt-hPAH were separated by size exclusion chromatography [4]. The fusion protein of the N-terminal fragment, MBP-(pep)$_{Xa}$-hPAH (Ser-2-Ser-110), containing the target sequence for factor Xa (IEGR) was expressed in the pMAL system in *E. coli* and isolated. The fusion protein was cleaved by the restriction protease at 0°C for 6 h, at a protease to substrate ratio of 1:100 (w/w). The cleaved fusion protein was gel filtrated on a Sephadex G-25 column (1.5×7 cm), equilibrated in 10 mM Na-HEPES, 0.2 M NaCl, pH 7.4 and then applied to a second column of amylose resin (2.5×8 cm), equilibrated in the same buffer, in order to remove MBP. The hPAH(Ser-2-Ser-110) protein was collected in the flow-through, and concentrated on Centriplus 3 concentrators (Amicon, MA), and further by freeze-drying. All chromatographic steps were carried out at 4°C.

2.4. Phosphorylation of the N-terminal domain

The cleavage of the fusion protein of wt-hPAH by factor Xa may result in variable amounts of a truncated form of the enzyme (Δ13-hPAH), which is not a substrate for PKA [4,5]. Thus, in order to phosphorylate both wt-hPAH and its N-terminal fragment at Ser-16 with a stoichiometry of 1 mol of P_i/mol subunit or domain, phosphorylation by PKA was performed on the fusion proteins prior to the cleavage by factor Xa [5]. The fusion proteins (2 mg/ml) were incubated with 8 μg/ml catalytic subunit of bovine heart PKA, 0.03 mM EDTA, 1 mM DTT, 63 mM $MgSO_4$, 4 mM $CaCl_2$, 0.5 mM [γ-^{32}P]ATP (or ATP) and 15 mM Na-HEPES, pH 7.5, at 30°C. Under these conditions, 0.9–1.0 mol phosphate was incorporated per hPAH subunit [5] or N-terminal fragment, hPAH(Ser-2-Ser-110). After phosphorylation the samples were gel filtered on a Sephadex G-25 column (1.5×7 cm), equilibrated in 10 mM Na-HEPES, 0.2 M NaCl, pH 7.4. Cleavage by factor Xa and isolation of phosphorylated hPAH(Ser-2-Ser-110) was performed in the same way as for the non-phosphorylated form (see above). In order to study the rate of phosphorylation for wt-hPAH and its N-terminal fragment, aliquots were taken out from the phosphorylation mixture at different times and the reaction stopped by addition of electrophoresis buffer containing SDS, followed by incubation at 95°C. Gels were analyzed by SDS-PAGE and autoradiography [5].

2.5. Infrared spectroscopy

Samples of hPAH for IR spectroscopy were prepared by dissolving the lyophilized protein in 20 mM Na-HEPES, 150 mM NaCl, pH or pD (H_2O- or D_2O-based buffers) 7.4, except for the samples of the N-terminal fragment in which the pH (pD) was originally 8.0 and it was adjusted to 7.4 after the protein was dissolved. Prior to the measurements, samples were centrifuged (10 min at 15 000×*g*) to remove any protein aggregates. The protein concentration was 14–18 mg/ml, except for the N-terminal fragment which was 4–5 mg/ml due to its reduced solubility. Samples were assembled in a thermostated cell between two CaF_2 windows with a pathlength of 6 μm (H_2O) or 50 μm (D_2O). Samples in deuterated buffer were left 3 h at room temperature to allow isotopic substitution of the protein exchangeable NH protons. Spectra were recorded in Nicolet 520 (D_2O samples) or Magna 550 (H_2O samples) spectrometers equipped with MCT detectors. A total of 450 (D_2O) and 1000 (H_2O) scans were accumulated for each spectrum, using a shuttle device. To study the thermal stability, samples were heated in steps of about 3°C in the interval of 25–85°C. After each heating step, the sample was left to equilibrate for 5 min and the corresponding spectrum recorded. Solvent subtraction, deconvolution, determination of band position and curve fitting of the original amide I band were performed as described [11].

2.6. Other methods

SDS-12% PAGE was performed at 12 mA (2.5 h), and the gels stained by Coomassie brilliant blue. Protein concentration was estimated [12] using as standard proteins purified fusion protein and isolated wt-hPAH, having the absorption coefficients A_{280} nm (1 mg ml^{-1} cm^{-1}) = 1.63 and 1.0, respectively [4]. Preparative and analytical size exclusion chromatography was performed at 4°C using a HiLoad Superdex 200 HR column (1.6 cm×60 cm) as described [1,4]. The catalytic activity of wt-hPAH and its truncated forms was measured as described [4].

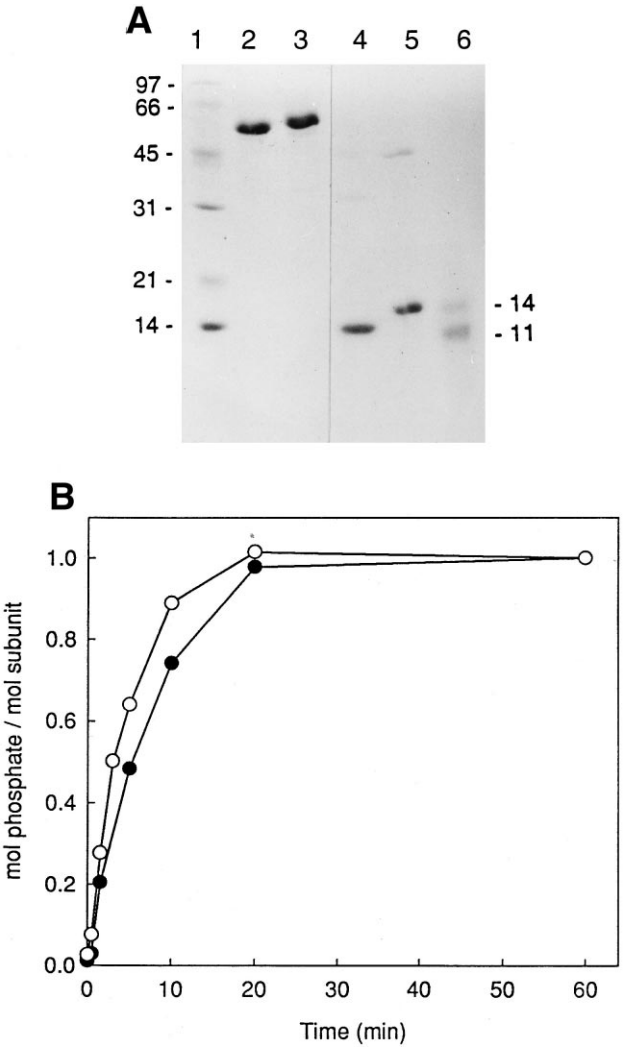

Fig. 1. Purification and phosphorylation of the N-terminal fragment of human phenylalanine hydroxylase. A: Purification and effect of phosphorylation on the electrophoretic mobility on SDS-PAGE. Lane 1, low molecular mass standards; lanes 2 and 3, non-phosphorylated and phosphorylated fusion protein MBP-(pep)Xa-hPAH(Ser-2-Ser-110), respectively; lanes 4 and 5, non-phosphorylated and phosphorylated N-terminal fragment hPAH(Ser-2-Ser-110), respectively; lane 6, extra low molecular mass standards: myoglobin peptides residues 1–131 (14 kDa) and residues 56–153 (11 kDa). B: Rate of phosphorylation of wt-hPAH (●) or N-terminal fragment, hPAH(Ser-2-Ser-110) (○). The fusion proteins were incubated with the catalytic subunit of PKA at 30°C in a phosphorylation assay mix, and aliquots were removed and tested for incorporated phosphate at the indicated times as described in Section 2.

3. Results

3.1. Isolation of the multiple forms of hPAH and phosphorylation of the N-terminal fragment hPAH(Ser-2-Ser-110)

The tetrameric and dimeric forms of wt-hPAH, the tetrameric truncated form hPAH(Asp-112-Lys-452), and the dimeric truncated mutant hPAH(Ser-2-Gln-428) were isolated to homogeneity [1]. The fusion protein MBP-(pep)$_{Xa}$-hPAH(Ser-2-Ser-110) was expressed in *E. coli*. Affinity chromatography yielded 3–5 mg of homogeneous protein per litre of culture (vs. 20–25 mg/l obtained for wt-hPAH) and the N-

terminal fragment was isolated following cleavage of the fusion protein by the restriction protease factor Xa (Fig. 1A). The fragment was devoid of catalytic activity, and on size exclusion chromatography it was recovered mainly in a monomeric form, although there were also high molecular mass aggregates that could be removed by centrifugation (data not shown). On phosphorylation by PKA its mobility on SDS-PAGE decreased, as also observed for its parent fusion protein (Fig. 1A). The apparent molecular mass increased from 55.4 to 57.6 kDa for the fusion protein and from 12.8 to 14.9 kDa for the isolated fragment on phosphorylation. A similar shift in the electrophoretic mobility following phosphorylation at Ser-16 is a characteristic feature of the human enzyme and is not observed for the rat enzyme [4,5]. A complete shift in electrophoretic mobility correlated with a stoichiometry of 1 mol of phosphate/mol of protein subunit, and the rate of phosphorylation by PKA can be followed by SDS-PAGE. The N-terminal fragment incorporated 1 mol phosphate/mol subunit at comparable rates to wt-hPAH (Fig. 1B), indicating a similar accessibility and folding around the phosphorylation site for both proteins.

3.2. Conformational properties of the different hPAH forms

The IR spectrum (recorded in D_2O buffer) of tetrameric wt-hPAH (Fig. 2A) revealed four band components in the amide I region (1700–1600 cm^{-1}) after deconvolution. The dominant band at 1654 cm^{-1} reflects a high content of α-helical structure. The band component at 1636 cm^{-1} indicates the presence of β-structure, although, as it has been described for human tyrosine hydroxylase (TH), it can also contain contributions from non-structured conformations [13]. The fact that the latter overlaps with the vibration of the helical component in H_2O (data not shown), made it possible to estimate its relative content by a comparison of the spectra recorded in both solvents. The increase of the band area of the 1636 cm^{-1} band component, and its upward shift (1–2 cm^{-1}) upon deuteration further reinforces that this component contains contributions from both types of secondary structures. The band at 1627 cm^{-1} has been assigned to protein segments in an extended configuration, with their peptide residues not forming intramolecular β-sheets, but rather hydrogen bonded to other molecular structures [11,14]. Finally, the component band at 1677 cm^{-1} has been associated to β-turns [15,16]. The secondary structure of wt-PAH as obtained from the combined values obtained in H_2O and D_2O media is shown in Table 1. Similar values were estimated for the dimeric form of wt-hPAH, which after isolation remains as 90–95% dimeric [1] (data nor shown).

The IR spectrum of the truncated protein hPAH(Asp-112-Lys-452), lacking the N-terminal domain, displayed the same

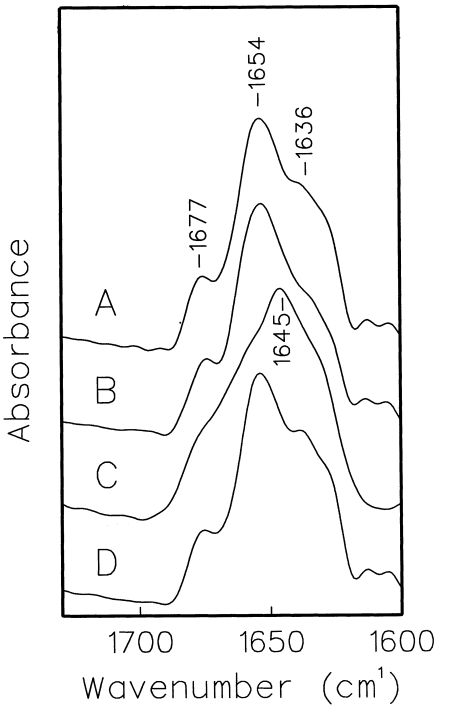

Fig. 2. Deconvolved infrared spectra of human phenylalanine hydroxylase (hPAH) and its truncated forms in deuterated buffer. A: wt-hPAH; B: hPAH(Asp-112-Lys-452), C-terminal domain; C: hPAH(Ser-2-Ser-110), N-terminal domain; and D: hPAH(Ser-2-Glu-428), stable dimer. All spectra were recorded at 25°C. Deconvolution was preformed using a Lorentzian line shape of 18 cm^{-1} and a resolution enhancement factor of 2.

number of band components at similar positions as wt-hPAH (Fig. 1B). However, the intensity of the band at 1636 cm^{-1} is significantly lower for the truncated protein, which can be interpreted as a decrease in the content of β- and/or non-regular structures upon removal of the N-terminal domain of the protein. In support of this interpretation is the fact that the relative intensity of the band components at 1655 cm^{-1} and 1635 cm^{-1} is almost identical in the spectra recorded in H_2O and D_2O. Taking this into account, the regulatory N-terminal domain is expected to be composed mainly of unordered structures with few segments of periodic secondary structure, which indeed was observed in the IR spectrum of this domain (Fig. 2C). The absorption maximum of the amide I band appears at 1645 cm^{-1}, a frequency that has been associated with unstructured protein conformations. The fact that deconvolution is not able to resolve the band components as clearly as in the other two forms of the enzyme, indicates that their bandwidths are larger, a character-

Table 1
Estimation of the relative amount (%) of different secondary structures in wild-type and truncated forms of human phenylalanine hydroxylase[a]

	hPAH (2–452)	hPAH (112–452)	hPAH (2–110)	hPAH P_i(2–110)	hPAH (2–428)
α-Helix	48	58	14	22	46
Extended structures (including β-sheet)	28	28	52[b]	52[b]	27
Unordered	12	2			14
β-Turns	12	12	34	26	13

[a]The values are rounded off to the nearest integer.
[b]These values include extended and unordered conformations.

istic property of flexible, fluctuating protein conformations [17]. Minor components at 1655 and 1630 cm^{-1}, revealed that this domain also contains some regular secondary structure elements. Although the N-terminal domain had a tendency to aggregate at concentrations higher than 5 mg/ml, no significant component bands at 1684 and 1619 cm^{-1}, corresponding to aggregated, denatured protein [18], could be observed in the spectrum of this domain, indicating that under the experimental conditions used (i.e. below 5 mg/ml), the fragment did not self-associate. The structural effects of phosphorylation of the N-terminal fragment at Ser-16 were also studied by IR spectroscopy. The sequence surrounding the phosphorylatable residue (Ser-16) has been found for many substrates of PKA and it has been predicted to lie within a β-turn [19]. The effects of phosphorylation of Ser-16 on the secondary structure are summarized in Table 1. As compared with the non-phosphorylated sample, it shows a higher (~8%) content of helical structure and a concomitant decrease in β-turn conformations.

The C-terminal 24 amino acids of hPAH consist of a heptad repeat in an amphipatic α-helix [20] as in rat TH, where it represents the tetramerization domain, involved in the formation of tetramers by domain swapping [6]. Removal of this C-terminal fragment generates a catalytically active dimer hPAH(Ser-2-Gln-428) [1]. The IR spectrum of this dimer shows the same spectral features as that of wt-hPAH (both dimeric and tetrameric forms), with regard to the number, position and intensity of their band components (Fig. 2D). Therefore, the secondary structure of the protein remains essentially unaltered upon tetramerization (Table 1). Interpretation of the observed differences between the molecular forms of hPAH based on percentage changes are not straightforward, since they contain different numbers of amino acids. To better visualize these differences other parameters such as band area ratios, which isolate two components and make them comparable between two samples, must be used (Table 2). The ratio α-helix/extended structures (1654/1636+1625) increases after deleting the 111 amino acid fragment at the N-terminal, as does the ratio α-helix/unordered (7-fold increase). It should be noted that the low solubility of the N-terminal fragment hampers data acquisition in H_2O medium. Therefore, the amount of unordered structure cannot be accurately determined. Nevertheless, from the data obtained it is clear that both band ratios mentioned above decrease significantly for this protein fragment.

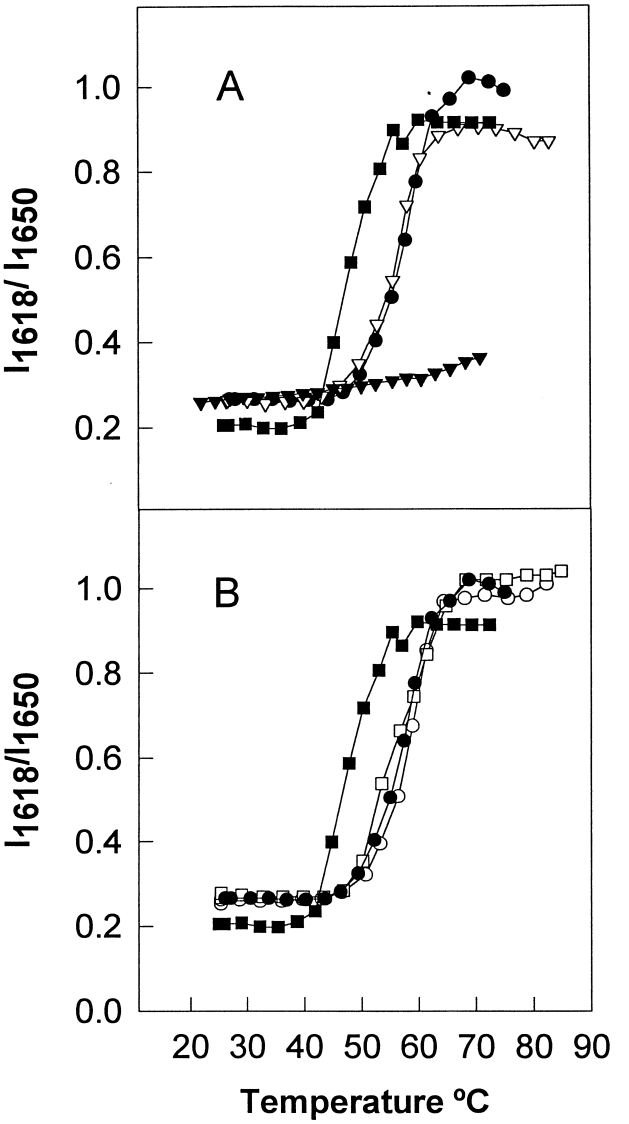

Fig. 3. Thermal stability of human phenylalanine hydroxylase and selected truncated forms of the enzyme. A: Ratio of amide I band intensity at 1618 cm^{-1} to that at 1650 cm^{-1}, in the absence of L-Phe, as a function of temperature for wt-hPAH (●); hPAH(Asp-112-Lys-452), C-terminal domain (■); hPAH(Ser-2-Ser-110), N-terminal domain (▼); and hPAH(Ser-2-Glu-428), stable dimer (▽). B: Temperature dependence of I_{1618}/I_{1650} for wt-hPAH (●, ○) and its C-terminal domain (■, □) in the absence (filled symbols) and presence (empty symbols) of 1 mM L-Phe.

3.3. Thermal stability studies

The thermal stability of the full-length protein and its truncated forms was also studied by IR spectroscopy. As observed for many soluble proteins [11,18], thermal denaturation of hPAH is accompanied by drastic changes in the amide I band contour. The most characteristic one is the appearance of two component bands at 1684 and 1619 cm^{-1} in the spectrum of the thermally denatured protein, that have been assigned to the aggregation of denatured protein molecules [18,21] (data not shown). From these spectral changes the thermal stability of the enzyme forms was estimated by following the intensity ratio of the amide I band at 1619 cm^{-1}

Table 2
Percentage ratios of different secondary structure elements of hPAH and its deletion mutants

	hPAH (2–452)	hPAH (112–452)	hPAH (2–110)	hPAH P_i(2–110)	hPAH (2–428)
α/extended	1.7	2.1	n.d.	n.d.	1.7
α/extended+unordered	1.2	1.9	0.3	0.4	1.1
α/unordered	4.0	29.0	n.d.	n.d.	3.3
α/turns	4.0	4.8	0.4	0.8	3.5

n.d., not determined.

and 1650 cm^{-1} (I_{1619}/I_{1650}) [13] (Fig. 3). The cooperative unfolding of tetrameric wt-hPAH shows a midpoint denaturation temperature (T_m) at 57°C, that decreases to 49°C upon removing the N-terminal domain (Fig. 3A). On the other hand, the thermal unfolding of the non-phosphorylated N-terminal regulatory domain shows a gradual, non-cooperative process which has also been observed for flexible protein conformations lacking stable tertiary structures [14,22] (Fig. 3A). The higher value of the amide I bandwidth at half-height obtained for this sample (51 cm^{-1}) as compared with those of wt-hPAH and hPAH(Asp-112-Lys-452) (42 cm^{-1}), is in agreement with this idea. In spite of the above mentioned increase in α-helical structure observed upon phosphorylation of the N-terminal domain, its thermal stability profile is similar to that of the non-phosphorylated sample (data not shown). The deletion of the tetramerization domain had no significant effect on the T_m (Fig. 3A), indicating that this domain does not play an important role in maintaining the thermal stability of the enzyme.

Incubation of wt-hPAH and the C-terminal domain hPAH(Asp-112-Lys-452) with L-Phe results in an activation of both enzyme forms [1], that is not accompanied by noticeable changes of their secondary structure (results not shown). However, the thermal stability of the truncated, but not that of the wild-type enzyme increases. The midpoint denaturation temperature of the former shifts from 49°C to 56°C, while that of wt-hPAH remains the same upon incubation with L-Phe (Fig. 3B).

4. Discussion

The three tetrahydrobiopterin-dependent aromatic amino acid hydroxylases have a highly conserved amino acid sequence at the C-terminal catalytic domain, and a less conserved sequence at the N-terminal regulatory domain which account for some of the catalytic and regulatory properties of the enzymes [23]. In the present study infrared spectroscopy has been applied to wt-hPAH and three truncated forms of the enzyme to elucidate the conformation of the catalytic and regulatory domains of the protein. The ability of the isolated domains to fold into native-like structures is demonstrated, among others, by (i) the C-terminal region (Asp-112-Lys-452) shows catalytic activity and forms tetramers [1], and (ii) the N-terminal fragment (Ser-2-Ser-110) is phosphorylated at the same stoichiometry and comparable rate as the wild-type enzyme by PKA, which changes its mobility on SDS-PAGE. The isolated domains of TH have also been shown to fold independently in a functional conformation [24]. Our results clearly demonstrate that the secondary structure elements of hPAH are segregated into two domains. While the catalytic domain is highly α-helical, the conformation of the N-terminal regulatory domain is more flexible and contains a higher relative content of turns and non-regular structures. The high content of α-helical structure of the C-terminal fragment is in good agreement with the secondary structure predicted using the PHD algorithm [25], that has given reliable data for the catalytic domain of TH [6]. The similar secondary structure and thermal stability of hPAH (present study) and TH [12] is in good agreement with the prediction that these proteins share a common overall fold [23].

The activation of PAH by incubation with its substrate (L-Phe) seems to induce changes in the tertiary and/or quaternary structure of the enzyme, probably resulting from homotropic cooperative interactions between the catalytic sites in the tetrameric enzyme [26]. This conclusion is further supported by an increase in volume of the tetrameric protein [27], the displacement of Trp-120 from a relatively hydrophobic to a hydrophilic (solvent) environment [28] and by a shift of the dimer:tetramer equilibrium towards the tetrameric form [4] upon binding of L-Phe. As seen from the present IR studies, these changes are not accompanied by measurable changes in the secondary structure. Moreover, it has been postulated that activation of PAH involves changes in the interactions between the catalytic and the regulatory domains which modify the accessibility of the active site for substrate binding. Thus, removal of the regulatory domain seems to expose the active site, relieving its inhibitory effect on the catalytic domain [1,29,30]. The existence of electrostatic interactions between the two domains which are changed on phosphorylation of the enzyme has also been postulated [31]. However, direct experimental evidence for the existence of such interactions is scarce. In this context, the lower thermal stability of the C-terminal domain (hPAH(Asp-112-Lys-452)) as compared to wt-hPAH, suggests that in the full-length protein the interaction between the two domains exerts a stabilizing effect. Accordingly, the displacement of the N-terminal domain on activation by the substrate may result in a destabilization of the enzyme which could be masked by the stabilizing effect of the substrate binding at the active site, as seen for hPAH(Asp-112-Lys-452) on incubation with L-Phe. Moreover, as shown here, the increase in the helical content of the N-terminal domain upon activation of the enzyme by phosphorylation could be involved in the modification of the interactions between domains which regulates access of substrates to the active site. A similar increase in α-helical content has been found upon phosphorylation of human TH, reinforcing their structural homology [13].

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