Urea-induced Denaturation of Human Phenylalanine Hydroxylase*  

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Human phenylalanine hydroxylase was expressed and purified from *Escherichia coli* as a fusion protein with maltose-binding protein. After removal of the fusion partner, the effects of increasing urea concentrations on enzyme activity, aggregation, unfolding, and refolding were examined. At pH 7.50, purified human phenylalanine hydroxylase is transiently activated in the presence of 0–4 mM urea but slowly inactivated at higher denaturant concentrations. Intrinsic tryptophan fluorescence spectroscopy showed that the enzyme is denatured through at least two distinct transitions. The presence of phenylalanine (τ-Phe) shifts the transition midpoint of the first transition from 1.4 to 2.7 M urea, whereas the second transition is unaffected by this substrate. Apparently the free energy of denaturation was almost identical for the free enzyme and for the enzyme-substrate complex, but significant differences in 

\[
\Delta G_{\text{d}} \text{d[urea]} \end{align}

were observed for the first denaturation transition. In the absence of substrate, a high rate of non-covalent aggregation was observed for the enzyme in the presence of 1–4 mM urea. All three tryptophan residues in the enzyme (Trp-120, Trp-187, and Trp-326) were mutated to phenylalanine, either as single mutations or in combination, in order to identify the residues involved in the spectroscopic transitions. A gradual dissociation of the native tetrameric enzyme to increasingly denatured dimeric and monomeric forms was demonstrated by size exclusion chromatography in the presence of denaturants.

Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, PAH, EC 1.14.16.1) is one of three enzymes constituting the pterin-dependent amino acid hydroxylase superfamily, all of which catalyze rate-limiting reactions of several metabolic pathways (1). In particular, loss of hPAH activity forms the disease PKU, phenylketonuria (PKU), and readily gives estimates of their stability (15). Activity measurements, turbidimetric measurements, fluorescence spectroscopy, and size exclusion chromatography (SEC) have been used to monitor conformational changes and oligomeric structure during unfolding of l-Phe-activated and non-activated hPAH.

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1 The abbreviations used are: PAH, l-Phe hydroxylase; hPAH, human PAH; wt-PAH, wild-type PAH; rPAH, rat PAH; hTPH, human tryptophan hydroxylase; DTT, dithiothreitol; IRS, infrared spectroscopy; SEC, size exclusion chromatography; GdnHCl, guanidine hydrochloride; wt, wild type; PKU, phenylketonuria; the abbreviations W120F/W326F and W187F/W326F represent mutant proteins, in which tryptophan residues were replaced by phenylalanine residues.
In order to localize conformational changes upon denaturation, tryptophan to phenylalanine mutants were included in these studies.

**EXPERIMENTAL PROCEDURES**

**Materials**—Guanidine hydrochloride (GdnHCl), potassium iodide, sodium chloride, sodium thiourea, EDTA, and ammonium Fe(II) sulfate were from Merck. (6) Tetrahydrobiopterin was purchased from Schiffl Laboratories (Switzerland). Dithiothreitol (DTT), catalase, and L-Phe were from Sigma, and urea was from Fluka. All reagents were of analytical grade.

**Expression and Purification**—Recombinant wt- and mutant hPAH were expressed in *E. coli* as maltose-binding fusion proteins, using the pMAL vector system, cleaved, and purified to homogeneity, in order to remove the tryptophan-rich fusion partner (8, 16). Enzyme concentrations were measured spectrophotometrically using ε_{280} = 1.00 mg⁻¹·ml⁻¹·cm⁻¹ for wt and 0.63 mg⁻¹·ml⁻¹·cm⁻¹ for the single tryptophan mutants (8).

**Enzymatic Activity**—PAH activity was measured as described by Martinez et al. (16), using an assay mixture containing 1 mM l-Phe, 0.1 mM Na-Hepes, pH 7.5, 0.2 mM NaCl, 1 mg·ml⁻¹·catalase, 0–8 mM urea, 2.0 μg of catalase, and 5 mM DTT. Enzyme concentrations in a final reaction volume of 100 μl and stopped with an equal amount of 1% (v/v) acetic acid in ethanol. The amount of L-tyrosine formed was determined using high performance liquid chromatography (17).

**Equilibrium Denaturation**—The enzyme samples were incubated in darkness at 25 °C for 18 h in 0.1 mM Na-Hepes, pH 7.5, and 0–8 mM urea. EDTA (1.25 mM) was added to prevent the quenching of fluorescence by metal ions (18). Buffer solutions were filtered through a 0.22-μm syringe filter before use.

**Fluorescence Measurements**—All data were obtained on a Perkin-Elmer 500 fluorimeter with a constant temperature cell holder with maximal stirring at 25 °C. Intrinsic tryptophan fluorescence was measured using excitation at 295 nm (slit 6 nm) and emission from 300 to 480 nm (slit 4 nm) with a scan speed of 240 nm·s⁻¹. Similarly, intrinsic tryptophan and tyrosine fluorescence were measured in the emission range 280 to 460 nm upon excitation at 275 nm. Protein concentrations were kept at less than 0.1 mg·ml⁻¹, in order to minimize the inner filter effect (ε₂₉₀ = 0.02). Fluorescence quenching experiments were performed by the stepwise addition of quenching solution (5 mM KI containing 10 mM Na₂S₂O₃) to prevent oxidation of iodide to I₂ (19) to the solution of denatured hPAH. The fluorescence quenching data were analyzed according to the Stern-Volmer and the modified Stern-Volmer equations (20).

**Aggregation**—Protein aggregation was estimated by measuring optical density at 340 nm, where soluble PAH has a low absorbance (1). Measurements were performed at 25 °C using a 8452A diode array spectrophotometer from Hewlett-Packard.

**Size Exclusion Chromatography (SEC)**—The molecular dimensions of native and denatured hPAH were estimated using an Amersham Pharmacia Biotech Superdex 200 HR 30/10 column and a Bio-Rad BioLogic HR chromatography system. The buffer was pumped at a flow rate of 0.50 ml·min⁻¹, and the column was calibrated using proteins with Stokes radii taken from the literature as compiled by Uversky (21) with Equation 6

\[ A_{295} = \sum s_i x_i \]  
\[ x_i = Q^{-1} \times x_i = K_i \chi_i Q^{-1} \]  
\[ K_i = e^{\Delta G_{i}^{D} / R T} \]  
\[ \Delta G_{i}^{D} = \Delta G_{i}^{D(0)} + m_i d \]  (Eq. 1–4)

Where i, j = 1 corresponds to the native state; i, j = 2 the intermediate state; and i, j = 3 the unfolded state, s, is the signal contribution of state i at the applied protein concentration; x is the molar fraction of component i at the denaturation concentration d, and S_{obs} is the observed fluorescence signal. K_{i} is the equilibrium constant of the transition i to j, and Q is the partition function of the system (1 = 1 + K_{d} + K_{2}K_{d}); R is the gas constant; T the temperature (in Kelvin); \Delta G_{i}^{D(0)} is the free energy of denaturation without denaturant present for the i to j transition; and m_{i} is the corresponding denaturation index in the linear extrapolation method (see Equation 4). Linear variations of x_{i} were included in the calculations by linear regression of observations at denaturation concentrations where more or less pure states were observed. Sigma Plot (Jandel Scientific Corp.) was used for fitting the three-state model to the observed data.

**RESULTS**

**Activity Measurements**—The activity of hPAH was measured after urea or GdnHCl denaturation, performed in the presence or absence of its amino acid substrate. As both denaturants had qualitatively similar effects on PAH activity, only the urea measurements are presented in Fig. 1. Depending on assay conditions, L-Phe is known to increase the specific activity of mammalian PAH severalfold when the enzyme is preincubated with this substrate for some minutes before catalysis (1). This activation of native hPAH is evident from Fig. 1 at low urea concentrations for 10 min in the presence of 0.5 mM L-Phe (△), expressed as the average of two separate measurements and for 10 (□) or 100 min (△), in the absence of L-Phe, both expressed as the means of eight separate measurements ± S.E. After preincubation for 10 or 100 min, either in the absence or presence of 0.5 mM L-Phe, the reaction was initiated by the addition of 5 μl of enzyme to 95 μl of assay mixture (see "Experimental Procedures"). Insert, kinetics of inactivation of wt-PAH (40 μg/ml) upon incubation at different urea concentrations (no L-Phe) plotted as remaining relative activity relative to non-denatured enzyme: no urea (○), 0.5 mM urea (□), 2.5 mM urea (△). Assay conditions were as described under "Experimental Procedures," but PAH was preincubated in presence of 1 mM L-Phe as described (18).

spectroscopic transitions, and the fraction of native, intermediate, and denatured enzyme was determined at each urea concentration. The linear extrapolation method (23) was used to calculate the thermodynamic stability in the absence of denaturant according to the Equations 1–4.
concentrations, but it is absent after 10 min incubation in 4 M urea. Upon denaturation in the presence of L-Phe, an almost linear loss in activity was observed with increasing urea concentrations, whereas in the absence of the amino acid a transient activation of hPAH at urea concentrations ranging from 0.5 M to 3 M was obvious. Still, the specific activity of urea-activated PAH was always lower than the substrate-activated enzyme. Interestingly, a marked minimum in the urea activation of PAH activity was apparent at urea concentrations ranging from approximately 1.5 to 2.5 M after only 10 min incubation and even more so after 100 min (Fig. 1).

Enzyme Aggregation—It has been reported that wt-PAH (16) and, in particular, mutant forms of the enzyme (8–12) have a tendency to aggregate in vitro. Using a diode array spectrophotometer, hPAH aggregation was estimated as turbidity at 340 nm at different urea concentrations (Fig. 2) and protein concentrations (data not shown). At urea concentrations where a significant aggregation was observed, the time course of the scattering intensity showed a sigmoidal shape (not shown), as is commonly observed (24, 25). For each experiment, the maximal rate of aggregation \((\frac{d[A_{340}]}{dt})_{\text{max}}\) was determined. As expected, increasing protein concentrations gave increasing rates and extent of aggregation. At constant protein concentration, the rate of PAH aggregation showed a distinct maximum between 1.5 and 2 M urea (Fig. 2). In the presence of 0.5 mM L-Phe, the empirical rate of aggregation was only 2% of the rate in the absence of the substrate (Fig. 2). However, turbidity data are not easily interpreted at a molecular level or in kinetic terms (24). Thus, turbidity measurements as performed here may not be suitable for the detection of low molecular weight aggregates. The aggregated protein could be dissolved in high concentrations of urea (data not shown), demonstrating the noncovalent interactions of the aggregates.

Fluorescence Measurements—As PAH is subject to aggregation in the presence of denaturants, fluorescence spectroscopy was selected for monitoring denaturation, since it can be performed at very low protein concentrations, where aggregation is minimized. As observed for activity measurements (Fig. 1), the changes in hPAH fluorescence induced by urea denaturation occurred slowly. Thus, all fluorescence measurements were performed after incubation for 18 h at 25 °C, which appeared sufficient to reach a steady signal.

Representative emission spectra obtained from equilibrium denaturation of nonactivated wt-hPAH are shown in Fig. 3A. Two different parameters, fluorescence intensity at 345 nm and the ratio of the fluorescence intensities at 355 nm versus 337 nm \((\frac{I_{355}}{I_{337}})\), were used to monitor denaturation. The ratio method had the advantage of being less susceptible to small variations in protein concentrations between experiments and reflects mainly the shifts in emission maxima upon denaturation, due to changes in polarity of the local environment surrounding tryptophan. However, as the observed fluorescence ratio is not strictly linear in terms of the molar fraction of the existing states, only the fluorescence intensity at 345 nm was used for model fitting (26). As shown in Fig. 4, two transitions were obvious for denatured non-activated and L-Phe-activated hPAH. Both spectroscopic parameters increased in a...

Fig. 2. Empirical rate of aggregation of wt-PAH at different urea concentrations. Turbidity \((A_{340})\) was measured as a function of time at different urea concentrations. The maximal rate of aggregation was determined for each curve. The concentration of PAH was 80 μg ml⁻¹ at all urea concentrations both in absence (○) and presence (□) of 0.5 mM L-Phe.

Fig. 3. Fluorescence spectra of wt and mutant hPAH. Tryptophan emission spectra, excitation at 295 nm, of equilibrium denatured, nonactivated wt-PAH at different urea concentrations (A); no urea (line 1), 4 M urea (line 2), and 7 M urea (line 3). Tyrosine-tryptophan emission spectra, excitation at 275 nm, of equilibrium denatured, nonactivated wt-PAH, W187F/W326F and W120F/W326F (B): wt-PAH at 4 M urea (line 1), W187F/W326F at 4 M urea (line 2), W120F/W326F at 4 M urea (line 3), wt-PAH at 7 M urea (line 4), W187F/W326F at 7 M urea (line 5), and W120F/W326F at 7 M urea (line 6). The protein concentration was 40 μg ml⁻¹.
linear fashion with changes in urea at concentrations flanking the transition regions, as is normally observed for pure states (26).

For hPAH incubated in the absence of amino acid ligand, increasing the urea concentration from 0.9 to 4 M resulted in a 75% increase in the tryptophan fluorescence intensity (Fig. 3A). This transition was accompanied by an approximately 2 nm red-shift in fluorescence emission maximum. Incubation with L-Phe prior to denaturation gave a more modest increase in fluorescence intensity (45%) and 6 nm blue shift in the emission maximum during the first transition. The two partially denatured states had similar fluorescence properties but a shift toward higher urea concentrations was observed in the midpoint for the transition of the activated native state to intermediate.

At higher urea concentrations PAH showed a loss in fluorescence intensity (approximately 25%), as well as a 10 nm red-shift in fluorescence intensity maximum both in the presence and absence of L-Phe. No differences in urea concentration of the denaturation midpoints were evident, which indicates that the ligand does not have any effect on the intermediate.

Fluorescence Quenching Studies—In order to address the fluorescence changes during unfolding and monitor the solvent exposure of indole moieties, fluorescence quenching studies by the ionic quencher iodide were performed. In addition, changes in indole fluorescence maximum may arise from other conformational changes, increasing the local environment polarity surrounding tryptophan (19). Quenching data recorded at several urea concentrations were plotted according to the Stern-Volmer equation (20). A downward deviation of the Stern-Volmer curves was observed in all experiments, as expected for a heterogeneous population of fluorophores. By using the modified Stern-Volmer equation, linear quenching plots were obtained. Only moderate changes in accessibility of the tryptophan residues to quenchers were observed during the first transition (up to 4 M urea), indicating minor opening of the core structure of hPAH, whereas a further increase of the urea concentration to 6.5 M resulted in an increased solvent exposure of the tryptophan(s) (Fig. 5). However, the theoretical limit of complete accessibility ($f_a = 1$) was not attained even in 8 M urea.

Denaturation studies were also performed on the W120F/W326F and W187F/W326F mutants of hPAH (in the following referred to as hPAH-W187 and hPAH-W120, respectively). Both showed similar fluorescence changes at higher urea concentrations compared with wt enzyme, i.e. a large red shift of the tryptophan emission maxima and a loss in tryptophan fluorescence intensity as well as an increased solvent exposure of the tryptophan residues (data not shown). The greater tendency toward aggregation, as well as differences in fluorescence changes during denaturation, indicate that the mutants are considerably less stable than the wt enzyme and can only give qualitative information on conformational changes of wt-PAH at a given urea concentration. As already shown by Knappskog and Haavik (8) at nondenaturing conditions, Trp-120 was the major contributor to fluorescence intensity also during denaturation and was especially dominating at urea concentrations below 4 M (data not shown).

At higher urea concentrations both mutants shifted their

![Fig. 4. Equilibrium denaturation of wt-PAH.](image1)

**Fig. 5. Solvent exposure of tryptophan residues during denaturation.** Fraction of tryptophans accessible to quenching by iodide at different urea concentrations (40 μg/ml protein concentration). Each point in the graph is calculated from the modified Stern-Volmer equation for a quenching experiment on the respective sample. The dotted curve is not based on calculations.
emission maximum to the same wavelength as the wild-type enzyme, but only hPAH-W187 seemed to increase its solvent exposure to the same extent as wt-hPAH (data not shown). The lower increase in $f_\text{c}$ for hPAH-W120 may be due to the presence of polar residues around the tryptophan other than the solvent. This is possibly the case for wt and hPAH-W187 too, as neither of them show complete solvent exposure despite their highly red-shifted spectra. However, the charged environment surrounding the tryptophans may also influence the accessibility to quenching by iodide. The incomplete accessibility of iodide is also compatible with the presence of some residual structure even in 8 M urea (see below). Experiments on the W120F/W187 double mutant have shown that replacing two tryptophan residues increases the tyrosine fluorescence at 4 M and especially at 7 M urea. Tryptophan-tyrosine emission spectra of wt and mutants has clearly shown that replacing two tryptophan residues increases the tyrosine fluorescence at 4 M and especially at 7 M urea. Tryptophan-tyrosine emission spectra of wt enzyme at 4 M urea were completely dominated by tryptophan fluorescence intensity. This could be due to an increased distance between the fluorophores, followed by lower energy transfer between tyrosine and tryptophan residues in the course of denaturation (Fig. 3B). Tryptophan fluorescence is insensitive to changes in the polarity of its environment (19), meaning that its emission maximum at 303 nm will be constant throughout the range of urea concentrations, irrespective of solvent effects and changes in its local side chain environment. Comparison between wt and mutants has clearly shown that replacing two tryptophan residues increases the tyrosine fluorescence at 4 M and especially at 7 M urea. Tryptophan-tyrosine emission spectra of wt enzyme at 4 M urea were completely dominated by tryptophan fluorescence (Fig. 3B). However, in 7 M urea a distinct emission peak appeared at 305 nm. This indicates that tyrosine fluorescence is not only superimposed by tryptophan but does appear as tryptophan fluorescence due to energy transfer or is due to the formation of nonfluorescent complexes. At least at 4 M urea, tyrosine fluorescence of the mutants was significant upon excitation at 275 nm and finally dominated over tryptophan fluorescence in the spectrum of Trp-187 at 8 M urea. It may be concluded that Trp-187 is a less effective quencher of tyrosine fluorescence than the other tryptophan residues.

**Simultaneous Tyrosine and Tryptophan Excitation**—At high urea concentrations tyrosine fluorescence was more pronounced for wt hPAH, due to a red shift of the tryptophan emission maximum and a decrease in tryptophan fluorescence intensity. This could be due to an increased distance between the fluorophores, followed by lower energy transfer between tyrosine and tryptophan residues in the course of denaturation (Fig. 3B). Tryptophan fluorescence is insensitive to changes in the polarity of its environment (19), meaning that its emission maximum at 303 nm will be constant throughout the range of urea concentrations, irrespective of solvent effects and changes in its local side chain environment. Comparison between wt and mutants has clearly shown that replacing two tryptophan residues increases the tyrosine fluorescence at 4 M and especially at 7 M urea. Tryptophan-tyrosine emission spectra of wt enzyme at 4 M urea were completely dominated by tryptophan fluorescence (Fig. 3B). However, in 7 M urea a distinct emission peak appeared at 305 nm. This indicates that tyrosine fluorescence is not only superimposed by tryptophan but does appear as tryptophan fluorescence due to energy transfer or is due to the formation of nonfluorescent complexes. At least at 4 M urea, tyrosine fluorescence of the mutants was significant upon excitation at 275 nm and finally dominated over tryptophan fluorescence in the spectrum of Trp-187 at 8 M urea. It may be concluded that Trp-187 is a less effective quencher of tyrosine fluorescence than the other tryptophan residues.

**Determination of Thermodynamic Parameters**—The most obvious interpretation of the denaturation profiles (Fig. 4) was a three-state denaturation for both substrate-activated and non-activated hPAH. The experimental data were fitted to this model as described under “Experimental Procedures” (black dots in Fig. 4A), and the results are summarized in Table I. As seen from the table, the transition midpoint of the first transition (state 1 to 2) was shifted upwards by 1.3 M urea in the presence of 0.5 mM L-Phe. However, no significant difference in $\Delta G^{\text{H2O}}_{12}$ values was obtained by these measurements, instead the shift was reflected in the $m_{12}$ values. The parameters of the second transition (state 2 to 3) were similar irrespective of the presence of L-Phe, within the errors given by these measurements.

**SEC of Denatured hPAH**—Based on the effects of urea on the elution volumes of PAH, a model for the stepwise dissociation and denaturation of this enzyme has been developed (Table II). Going from no urea to high denaturing conditions, the chromatograms were resolved into several components by adding new ones, preferably according to a two-state model for each transition (fluorescence data), whenever the components resolved at lower urea concentration did not suffice in the fitting. However, the interpretation of the SEC data was not unequivocal, due to the multiple oligomeric forms observed and in some cases the overlapping retention times of these species.

In the absence of L-Phe and urea, the majority of hPAH eluted with a volume corresponding to the tetramer, with a minor proportion corresponding to the octamer and dimer species (Table II and Fig. 6). By addition of L-Phe, the oligomeric state was shifted toward tetrameric and octameric PAH, as has previously been reported (1, 16). The observed tetrameric sizes for both nonactivated and substrate-activated hPAH (with Stokes radii of 53 and 54 Å, respectively) were almost identical to the values of previously reported SEC measurements of rat PAH (27) (55 and 57 Å, respectively).

The presence of low urea concentrations had a destabilizing effect on tetrameric and octameric PAH in favor of the dimer both in the absence and presence of L-Phe (Table II). However, in the presence of the substrate the transition to dimer appeared at higher urea concentrations (between 3 and 4 M urea). Below 4 M urea, components of both native and partially denatured dimer and tetramer could be resolved. The partially denatured species had $R_g$ values typically 4 Å larger than the corresponding native oligomer (Table II).

In the presence of 1–3 M urea, a slowly eluting component was observed for both activated PAH and more so for nonactivated enzyme (Fig. 6). The retention time for this component corresponds to a Stokes radius of 32 and 34 Å for nonactivated and activated PAH, respectively. This is approximately the expected size of monomeric PAH, as calculated from observed sizes of the native tetramers.

At high concentrations of urea a main component was of similar size as native octameric PAH. Further addition of denaturant (3 M GdnHCl) further contributed to an increase of the Stokes radius to 75 Å. At 6 and 7 M urea, small amounts of partially denatured dimer could be detected. This species seemed to increase in size at higher urea concentrations (Table II). The presence of 10 mM DTT had no effect on the calculated Stokes radius of PAH in the presence of denaturants.

**Reversibility of Transitions**—Partial reversibility (50–90%) of the second spectroscopic transition was achieved by diluting the enzyme from 3 M urea to 4–7 M final concentrations of urea (data not shown). However, more detailed studies are needed to determine whether further reversibility of PAH denaturation is possible.

**DISCUSSION**

**Activation and Denaturation of PAH**—The enzyme activation observed upon exposure to moderate concentrations of
urea or GdnHCl was similar to findings on the rat (r) enzyme (28), although rPAH was activated to a much larger extent. Parniak (28) has suggested that low concentrations of denaturant provoke conformational changes similar to those noted upon substrate-induced activation and reflect the denaturation of an “inhibitory domain” (28). Although this is an interesting hypothesis, spectroscopic data presented here clearly show that these processes are distinct. Instead of the 10 nm red shift and 15% increase in quantum yield observed by L-Phe activation (8), a modest 2 nm red shift and an 80% increase in fluorescence intensity was observed going from 0 to 3 M urea. Furthermore, the urea-treated enzyme had fluorescence spectra clearly different from those of the N-terminal deletion mutants (2), which have a diminished or abolished inhibitory effect of the N-terminal domain.

Partial Denaturation of hPAH—The activity measurements in the absence of urea show a partial inactivation of hPAH during the long incubations used in these studies. However, loss of activity does not seem to be reflected in the fluorescence measurements. Furthermore, SEC experiments at nondenaturing conditions show that PAH retains many of its native properties after such periods of time, including its compactness and oligomer equilibria. Partial denaturation of hPAH occurs between 0.7 and 2.5 M urea in the absence of L-Phe and between 1 and 4 M urea in the presence of this substrate, as reflected in the fluorescence intensities at 345 nm and by the fluorescence intensity ratios (Fig. 4). Partially denatured tetrameric and dimeric PAH were found both in the presence and absence of L-Phe. However, it was not possible to explain the chromatograms by pure two-state transitions, as some volume changes were evident from the appearance to the disappearance of the partially denatured species (Table II). Thus, some degree of variability is likely for the partially denatured states. The appearance of variable states in solvent denaturation studies has been reported frequently (22, 29, 30). The tryptophan quenching studies on PAH support the above observations: a minor volume increase during the first transition (relative to the second) and some degree of variability of the intermediate state.

The large effect on Trp-120 fluorescence as well as the activation of PAH is consistent with a denaturation of the N-terminal domain at low urea concentrations. The crystal structure of dimeric rPAH (residues 19–427) (31) shows a covering of the active site by the far N-terminal residues, which also may prevent efficient binding and enzymatic conversion of L-Phe. However, a partial denaturation of the N-terminal domain is unlikely to be causing the observed shift in oligomeric structure toward dimeric (and some monomeric) PAH below 4 M urea. Deletion mutation studies on hPAH (2) have shown that removal of the N-terminal does not dissociate the tetramer into dimers. Pure dimeric PAH was generated when residues 429–452, corresponding to the a-helix (a14) in the tetramerization motif (5), were deleted. Thus, the observed effects of urea could be due to conformational changes at the far C terminus during partial denaturation of hPAH. The appearance of a partially denatured tetramer for both nonactivated and activated PAH suggests that alterations in the C terminus require higher concentrations of urea than the suggested N-terminal alterations.

Turbidity measurements showed that the rate of aggregation is dependent on protein (not shown) and urea concentration (Fig. 2). The increased rate of aggregation at intermediate urea concentrations coincides well with the observed decrease of
activation observed between 1 and 3 M urea. However, as the activity at 2 M urea after 100 min incubation does not markedly differ from that at non-denaturing condition, aggregation probably does not influence the activity of PAH to a large extent. The identification of the aggregating species is not a trivial task. In the SEC experiments, aggregation was evident in the presence of 4 M urea, where only the denatured dimeric PAH was found. Whereas this is a likely candidate for an aggregating species under these conditions, other partially denatured forms of the protein probably also aggregate.

**Attempted Complete Unfolding of PAH—Denaturation of the partially denatured dimer was observed above 4.5 M urea and was, by fluorescence intensity measurements, apparently completed above 6.5 M urea. However, a further increase in volume is observed above this urea concentration. In fact, even in the presence of 8 M urea supplemented with 1.5 M GdnHCl and 10 mM DTT, the unfolding is incomplete, which shows the high degree of variability of this state. Only monomeric PAH can be expected to exist at such denaturing conditions. Hence, a concomitant unfolding of dimeric and dissociation to monomeric PAH is suggested. A sequential dissociation and unfolding would be expected to give monomeric non-unfolded components at 4–6 M urea, which is not observed.

An approximate 30-Å increase in Stokes radius is observed between 4 and 6 M urea, with a further 10-Å increase at 8 M urea supplemented with 3 M GdnHCl. This represents a rough 12× volume increase of the monomer relative to that at non-denaturing conditions (estimated as one-fourth of the observed tetramer volume). Such a large volume increase is not expected for a protein with a chain length of 439 amino acids. Monomer volume ratios above 8 between observations in 6 M GdnHCl and native conditions are common even for small monomeric proteins (21).

The above discussion suggests a minimal three-state model for the denaturation of hPAH. However, in their study on the thermal stability of hPAH using IRS, Chehin et al. (6) reported only one transition for wt-PAH. We suggest that their observation corresponds to the second transition, or the unfolding of PAH in this study, and that possible intermediates remain undetected as the IRS study focused on changes in α-helix content.

**Calculations of the Thermodynamic Parameters—**A three-state model was assumed for the unfolding of hPAH, as this adequately explained the observed fluorescence changes. However, the complexity of the changes in oligomeric structure during denaturation makes it virtually impossible to incorporate quantitatively all these effects into the denaturation model. Thus, the model fitted to the fluorescence data does not consider aggregation or changes in oligomeric structure. Because of this, the calculated thermodynamic parameters (Table I), as well as the apparent cooperativity, would be expected to depend on experimental conditions, in particular protein concentrations. Such deviations from ideality could influence the presented thermodynamic parameters in various ways. Furthermore, our preliminary experiments have not shown complete reversibility of the spectroscopic transitions. Thus, we are dealing with a pseudo-equilibrium system for which micro reversibility has been assumed.

As shown, despite the approximate 1.3 M shift in C_{m12} between nonactivated and 1-Phe-activated PAH, there seems to be little difference in their free energy of denaturation. Instead, the C_{m12} shift is reflected in the different m_D values, which is approximately 2-fold larger for the nonactivated compared with the activated enzyme. Based on a model analogous to Gibbs absorption isotherm, it has been suggested that the m_D value in the linear extrapolation method is proportional to the surface area exposed during denaturation (ΔA) (32). However, this relationship does not seem to apply for the partial denaturation of PAH, as both nonactivated and activated PAH have similar Stokes radii at 4 M urea and similar native structures. There are, however, a number of differences between the non-activated and activated enzymes in the transition regions, which may substantially influence the observed thermodynamic parameters, including their oligomeric structures and aggregation properties. Furthermore, the interpretation of the m_D value in terms of ΔA has been claimed to be oversimplified (30).

The parameters for the second transition were more or less equal for activated and nonactivated enzyme, as expected for the unfolding of equal intermediate states. However, the variable nature of the unfolded state might play a significant role when extrapolating the ΔG^{den} to nondenaturing conditions. Lack of nonlinearity in the extrapolation method might give erroneous estimates of ΔG^{den}23, a weakness criticized in the literature (30, 33, 34).

**PAH Stability and Its Importance in PKU—**Several reports exist on PAH mutant proteins, many of which are mutations associated with PKU. Many of the proteins show a high degree of instability and susceptibility toward aggregation and degradation (8, 9, 10, 11, 12, 35). The low stability of PAH toward partial denaturation and the observed aggregation at low urea concentrations could imply a role of this state in the understanding of PKU-related mutations (36). However, thorough denaturation studies have not been performed on any PKU-associated mutants, and these proteins may behave differently. Furthermore, several technical problems, including protein aggregation, must be resolved before such comparable analysis can be performed.

Recent studies on protein stability and engineering point toward the importance of the denatured state ensemble for understanding the native state stability (31, 32, 37). Searching for causes of decreased (or increased) stability of mutants in the native structure alone will probably fail in most cases, because such a reasoning implies that the denatured states behave as random coils in vivo, a hypothesis that has been negated by numerous observations (30, 38, 39). In particular, the denatured states of PAH have been shown to be fairly compact and hence contain many native-like interactions. As shown here, a complete random coil of PAH was not found even in 8 M urea supplemented with 3 M GdnHCl and 10 mM DTT.

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