Different stabilities and denaturation pathways for structurally related aromatic amino acid hydroxylases

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Abstract We have compared the urea stability of the human aromatic amino acid hydroxylases (AAAHs), key enzymes involved in neurotransmitter biosynthesis and amino acid homeostasis. Tyrosine-, tryptophan- and phenylalanine hydroxylase (TH, TPH and PAH, respectively) were transiently activated at low urea concentrations and rapidly inactivated in >3 M urea. The denaturation of TH occurred through two cooperative transitions, with denaturation midpoints of 1.41 ± 0.06 and 5.13 ± 0.05 M urea, respectively. Partially denatured human TH (hTH) retained more of its secondary structure than human PAH (hPAH), and was found to exist as tetramers, whereas hPAH dissociated into dimers. Furthermore, the urea-induced aggregation of hPAH was 100-fold higher than for hTH. These results suggest that the denatured state properties of the AAAHs contribute significantly to the stability of these enzymes and their tolerance towards missense mutations.

Keywords: Aromatic amino acid hydroxylase; Tyrosine hydroxylase; Protein stability; Urea denaturation; Aggregation

1. Introduction

The aromatic amino acid hydroxylases (AAAHs); phenylalanine-, tyrosine- and tryptophan hydroxylase (PAH, TH and TPH) have similar domain structure/organization, but different regulatory properties (for review see [1,2]). The AAAHs have important roles in normal amino acid metabolism and neurotransmitter biosynthesis, but have also been implicated in various human disorders. For PAH, more than 400 different human mutations have been discovered, most of which are associated with the severe metabolic disorder phenylketonuria (http://data.mch.mcgill.ca/pahdb_new/). In contrast, very few mutations have been found in the TH gene and no definite mutations have been reported for TPH. Most of the known PAH mutations cause a dramatic increase in the rate of enzyme aggregation and degradation [3], whereas at least some of the TH mutations appear to be kinetic variants, with little effect on protein stability [4]. Thus, it can be speculated whether the AAAHs have fundamentally different stabilities and tolerances towards missense mutations, despite their overall structural similarities.

Several investigations on TH stability have been performed, including the effects of enzyme phosphorylation [5–7]. However, only the thermal stability of the enzyme was examined and conflicting results have been reported. Previously, we have studied the solvent-induced denaturation of human PAH (hPAH) [8], and here we apply intrinsic fluorescence, circular dichroism (CD), size exclusion chromatography (SEC), enzymatic activity and light scattering measurements to get comparable results for human TH1 (hTH1). The data presented here indicate that although hPAH and hTH1 share many structural and functional properties, they denature through intermediates with different physical properties. As purified human TPH1 (hTPH1, peripheral type [9]) rapidly aggregates even in the absence of denaturants [10], the investigations on TPH stability were limited to activity studies.

2. Materials and methods

2.1. Materials

Sodium chloride, EDTA and iron(II) sulfate were from Merck. (6*R*)-L-Erythro-5,6,7,8-tetrahydrobiopterin was purchased from Schircks Laboratories (Switzerland). Dithiothreitol (DTT), catalase, HEPES and thrombin were from Sigma, and urea from Fluka. 3,5-[^{3}H]Tyrosine and [γ-^{32}P]ATP, Superdex 200 HR10/30 and the PC 3.2 column were from Amersham Biosciences. All reagents were of analytical grade.

2.2. Expression and purification of hydroxylases

Full-length hTH isoform 1 (hTH1; $A_{280}^{1\%} = 10.4$ cm^{-1} [11]) was expressed in *E. coli* and purified as described [12], whereas the purifications of hPAH from *E. coli* and hTPH1 (peripheral type) from *Pichia pastoris* were performed as described [10,13].

2.3. Urea denaturation and activity measurements

hTH1 and hTPH1 were preincubated with stoichiometric amounts of $FeSO_4$ and then exposed to different concentrations of urea for 10 min, 25 °C, before aliquotes were removed and immediately assayed for remaining activity, as previously described [14]. The following denaturing conditions were used: 0–7 M urea, 50 mM HEPES (pH 7.50), 200 mM NaCl and an enzyme concentration of 0.80 μM subunit.

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Abbreviations: AAAH, aromatic amino acid hydroxylase; TH, tyrosine hydroxylase; hTH, human TH; PAH, phenylalanine hydroxylase; TPH, tryptophan hydroxylase; CD, circular dichroism; LEM, linear extrapolation method; SEC, size exclusion chromatography; DTT, dithiothreitol

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2.4. Urea denaturation and fluorescence measurements

TH was preincubated with stoichiometric amounts of $FeSO_4$. Denaturation was performed in darkness in 0–7.2 M urea, 50 mM Na–HEPES (pH 7.5), 200 mM NaCl for 18 h, at 25 °C using a protein concentration of 44.8 $\mu g\,ml^{-1}$ (0.80 μM subunit concentration), prior to fluorescence measurements. All data were obtained on a Perkin–Elmer 50B fluorimeter as previously described [8].

2.5. Size exclusion chromatography

The oligomeric forms of hTH1 and hPAH during urea denaturation were analyzed by SEC. The samples were run on a Superdex 200 PC 3.2 column, connected to a Shimadzu HPLC system with UV absorbance detection at 280 nm. The column was calibrated and the chromatograms analyzed as described previously [8].

2.6. Light scattering measurements

The degree of protein aggregation was measured at 25 °C as light scattering, using a fluorimeter (Perkin–Elmer 5OB). Excitation and emission wavelengths were 295 and 300 nm, respectively, with slits of 3 and 4 nm. TH was incubated in the presence of stoichiometric amounts of $Fe(II)SO_4$ before addition to the denaturation assay and light scattering measurements. Protein concentrations of 80 and 89.6 $\mu g\,ml^{-1}$ were used for hPAH and hTH1, respectively (1.60 μM subunit concentration).

2.7. Circular dichroism measurements

The CD spectra of TH at different urea concentrations were determined at similar conditions as described above, except that 10 mM KH_2PO_4 and 150 mM KF were used instead of HEPES and NaCl. Control experiments were performed to verify that the fluorescence spectra and gel filtration profiles were identical in phosphate and HEPES buffers. The thermal stability of samples was measured at 222 nm, with 60 $°C\,h^{-1}$ increase in temperature.

3. Results

3.1. Activation of AAAHs at low urea concentrations

After 10 min incubation at 25 °C, the inactivation of hTH1 and hTPH1 was almost complete at 3.5–4 M urea (Fig. 1), and the reduction of activity was very steep with almost complete loss of activity going from 3.0 to 3.5 M urea. Interestingly, we found a temporary activation of both TH and TPH at low urea concentrations ($\leqslant 1$ M urea), very similar to hPAH [8]. Further experiments with incubation of TH at higher concentrations of urea for shorter time periods showed enzyme activation up to 3 M urea, but that the rate of inactivation was greatly increased. The maximal activation of the hydroxylases by urea varied from 3% to 50% between different enzyme batches and decreased after repeated freezing and thawing, indicating that it is dependent on the native conformation of the enzyme.

3.2. Spectroscopic changes during denaturation of hTH1

The denaturation of hTH1 monitored by intrinsic protein fluorescence was very similar to what we have previously reported for hPAH [8]. The tryptophan fluorescence intensity of hTH increased (76%) in 3.5 M urea, and then decreased again (25%) at higher urea concentrations, corresponding to two cooperative transitions (Fig. 2). We have previously observed a 2 nm red-shift of the emission maximum for hPAH during the first transition [8], but for hTH1 it remained unchanged (342.5–343.0 nm). However, by complete denaturation the emission maximum was red-shifted to 351 nm, as found for bovine TH [11].

In order to investigate whether the two transitions reflected major changes in the protein conformation, such as changes in secondary structure or domain denaturation, we measured the CD spectra for hTH1 at different concentrations of urea (Fig. 3A). In the absence of denaturant, two characteristic minima at 208 and 220 nm and a local maximum at 215 nm were apparent. However, these were lost during the first transition, with a concomitant loss of CD signals between 210 and 245 nm. Typical spectra of random coil peptides were not observed even at 7.5 M urea. For comparison, we also measured the CD spectra for hPAH at corresponding concentrations of urea (Fig. 3B). The CD signal from hPAH was lower

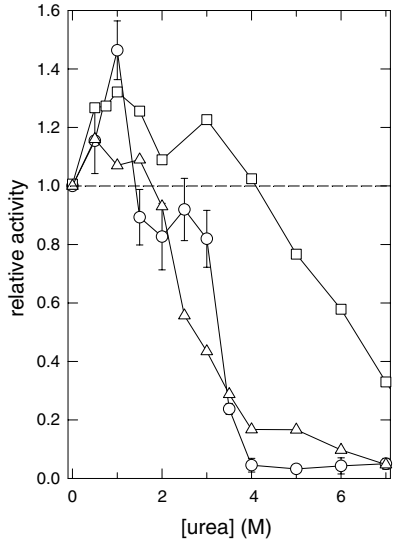

Fig. 1. Enzyme activity in the presence of urea. The loss of hTH1 (○), hPAH (□) and hTPH1 (△) activity at different urea concentrations was measured after 10 min incubation at 25 °C. Values are presented as ±S.D. ($n = 3$), or as an average of two different experiments. The values given are relative to 450, 740 and 10.7 $nmol\,min^{-1}\,mg^{-1}$ for hTH1, hPAH and hTPH1, respectively. The data for hPAH are from Kleppe et al. [8].

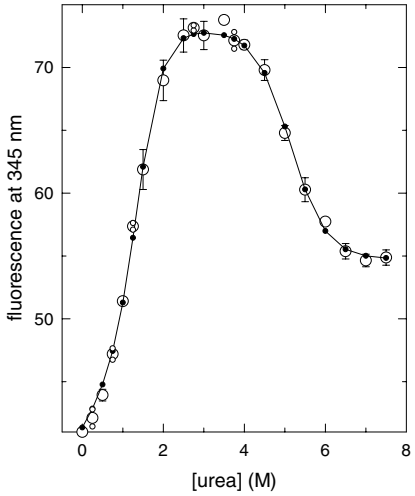

Fig. 2. Urea denaturation of hTH1. After denaturing as described in Section 2, the fluorescence intensity at 345 nm was measured for each sample. The data points are averages of two to three measurements, the S.D. are given for three measurements, and both measurements are shown for data points where only two exist (small open circles). The best fit to the data is shown in black.

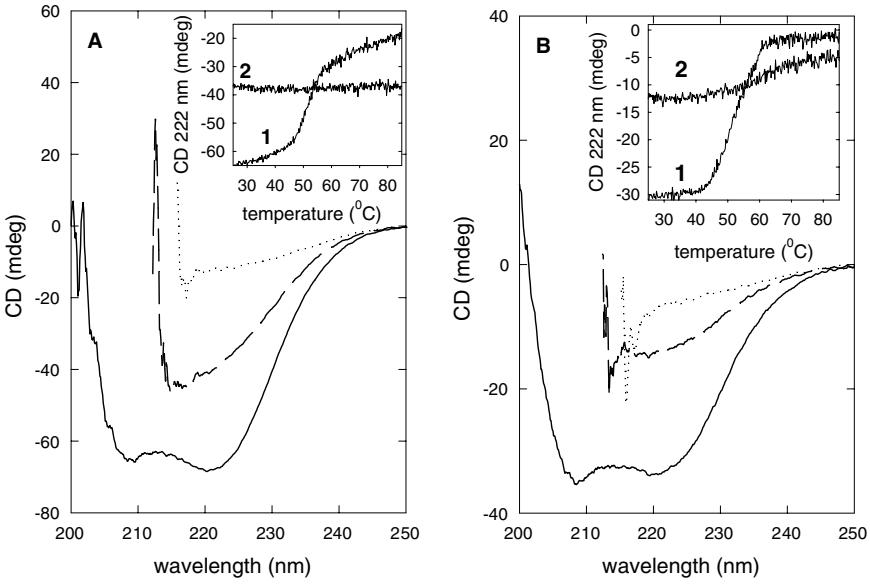

Fig. 3. Circular dichroism of denatured hTH1 and hPAH. The CD spectra of hTH1 (A) and the CD spectra of hPAH (B) are given at zero (—), 3.5 (– – –) and 7.5 M urea (···), 18 h incubation at 25 °C. *Insets:* The thermal denaturation of hTH1 (A) and hPAH (B) at zero (1) and 3.5 M (2) urea (18 h incubation at 25 °C) is followed at 222 nm.

than for hTH1, partly due to its smaller molecular size (0.8 μM subunit concentration was used for both). Similarly to TH, native PAH displayed two local minima (208 and 220 nm) and a local maximum (215.5 nm).

The fluorescence data (Fig. 2) were used to calculate the apparent free energy of denaturation for each transition by using the linear extrapolation method (LEM, [15]) as previously described [8]. The thermodynamic values calculated show considerably lower apparent free energy of denaturation for hTH1 (11.13 ± 0.56 and 29.1 ± 2.1 kJ mol^{-1}, first transition and last transition, respectively) than previously estimated values for hPAH (21 ± 2 and 57 ± 5 kJ mol^{-1}, first transition and last transition, respectively [8]). However, only for the last transition did the different midpoints of denaturation, i.e., 5.1 and 5.7 M, reflect the different free energy values. The corresponding denaturation index values for hTH1 for each transition were estimated to be -7.89 ± 0.42 and -5.67 ± 0.41 kJ liter mol^{-2}. All values are given ±S.E. obtained from the nonlinear regression analysis.

Denaturation of proteins by temperature and urea occurs by different mechanisms, and the results are not always comparable. This is also true for the AAAHs studied here. Thus, the CD spectra of hTH and hPAH after complete thermal denaturation were stronger than after denaturation at 25 °C in 7.5 M urea, suggesting that urea denaturation gives more unfolded states. These observations may be relevant when comparing results obtained by thermal and solvent denaturation of the AAAHs.

3.3. The tetrameric structure of hTH1 is more stable than for hPAH

It is well documented that under physiological conditions TH exists as a tetramer, whereas native PAH exists in an equilibrium between tetramers and dimers [8]. Our SEC data were in accordance with this, showing a single molecular species of native hTH1 with a Stokes radius of 59 Å. The effect of denaturation on the oligomeric structure of the AAAHs was studied by analyzing the urea denatured proteins by SEC. This analysis showed differences between hPAH and hTH1. For hPAH, the chromatograms were resolved into denatured and native tetramers and dimers, where only the denatured dimer form was detected after the first transition (2 M urea), as reported previously [8,16]. However, in 2 M urea hTH1 still existed as a (denatured) tetramer with an increased Stokes radius (65 Å) compared to the native enzyme. Further increase in the urea concentration above 4 M resulted in formation of unfolded monomeric forms for both hPAH and hTH1, with apparent molecular dimensions exceeding even their native tetrameric forms (hTH1 Stokes radius 69 Å in 8 M urea). Further addition of denaturant increased the apparent size of the denatured species of both hTH1 and hPAH, as expected for a gradual unfolding to random coils.

3.4. Different aggregation of partially denatured hTH1 and hPAH

During denaturation hydrophobic residues may be exposed to the solvent and cause protein aggregation, in spite of the generally solvating properties of urea. We have previously reported such behavior for hPAH and for comparison we investigated the aggregating properties of hTH1 during urea-induced denaturation. We found only modest changes in the aggregation of hTH1 at different urea concentrations (Fig. 4B), and the increase in light scattering was almost linear with time, as opposed to the typical sigmoid curves observed for hPAH (Fig. 4A and [8]). The maximal rate of aggregation for hTH1 was at 3 M urea, compared to about 1.5 M for hPAH (Fig. 4A and [8]). In order to get quantitative estimates of the rate of aggregation, we applied linear regression analysis to the light scattering curves in the steep sigmoid area and to all data points for linear light scattering curves. Compared to hTH1, hPAH displayed a strong tendency to aggregate, with the maximal empirical rate of aggregation being 100-fold higher than that of TH (Fig. 4C).

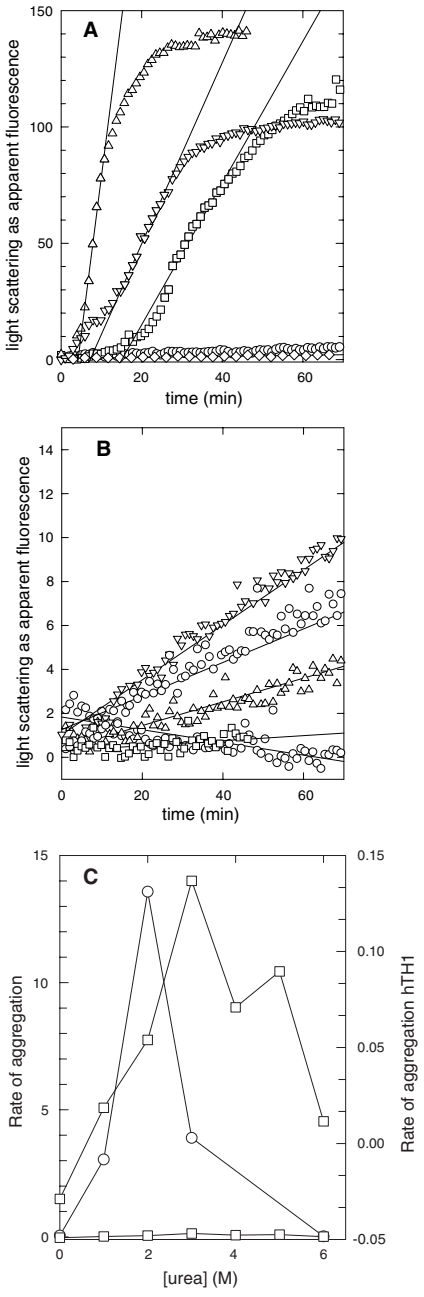

Fig. 4. Protein aggregation. Light scattering measurements were used to monitor the aggregation of hPAH (A) and hTH1 (B) at different concentrations of urea as described in Section 2. For (A), the increase in light scattering is shown at zero (○), 1 M (□), 2 M (△), 3 M (▽) and 6 M (◇) urea. The regression line in the steepest area of the curves is used as an empirical estimate of the rate of aggregation. Similarly, for hTH1 (B) the light scattering is shown at zero (○), 1 M (□), 2 M (△), 3 M (▽) and 5 M (○) urea. (C) The slope of each regression curve for hPAH (○) and hTH1 (□) is used to visualize the difference in rate/degree of aggregation. The data for hTH1 displayed at two different scales, the left axis corresponding to the scale for the hPAH data.

4. Discussion

In this study we compare the solvent stability of the structurally related AAAHs, revealing both similarities and important differences. The denaturation of such multi-domain oligomeric proteins is expected to be a complicated process, where the possible denaturation pathways depend on the intrinsic stabilities of the domains and domain–domain interactions both within each subunit and between different subunits. Together with previous studies on the denaturation of these enzymes, we show that these properties are important determinants of their stability and pathway of denaturation.

The observed activation at low denaturing conditions is probably related to common domain organization of the AAAHs, and several agents which activate the AAAHs exert their effects through the N-terminal regulatory domain [17–21]. The structure and stability of these intermediately denatured proteins are not presently known, however, it seems probable that some structural changes involving the N-terminal regulatory domain are occurring even at low urea concentrations, and that the activation seems to reflect a transiently populated intermediate. Experiments on the N-terminal truncated enzymes support this, and several studies by thermal denaturation of hPAH have shown that denaturation of the N-terminal domain occurs prior to other major structural changes [22]. In addition, urea denaturation studies on tryptophan to phenylalanine hPAH mutants suggest that major structural changes occur in the N-terminal of the enzyme [8].

However, for hPAH, we have found that the first transition not only involves the unfolding of the N-terminal domain, but also dissociation of the tetramer to a dimeric N-terminal denatured hPAH. Here we report that hTH1 keeps its tetrameric structure throughout the first transition, suggesting that the C-terminal oligomerization domain of hTH is more stable than for hPAH. An alternative explanation would be that some of the tetrameric hPAH disappeared by aggregation, but the CD data support more residual secondary structure of the partially denatured hTH than of hPAH. In fact, these data suggest that for both enzymes more secondary structure is lost during the first denaturation transition than can be attributed to the N-terminal domain alone. Thus, it seems that lower energy is needed to partially denature hTH1, but the state reached contains more residual structure as compared to hPAH.

For several decades the LEM method of Greene and Pace has been applied to get estimates of the thermodynamic stabilities of proteins. However, even in the recent literature we find no clear consensus on how this method should be applied to oligomeric proteins. For that reason, we have used the simplest approach where the oligomeric structure is not considered, which means that the values obtained depend on the conditions chosen. Thus, we used equal subunit concentrations for hTH1 and hPAH to get comparable results. The lower apparent stability of hTH1 than for hPAH is in agreement with previous findings on the thermal stabilities of TH and PAH [5,22,23]. However, according to the CD data (inset of Fig. 3A and B), thermal and urea denaturation is different for TH and PAH.

One of the most remarkable differences between hTH1 and hPAH was the distinct pattern of aggregation of these proteins observed during denaturation, which reflects differences in structures and properties of their denaturing intermediates. Due to the similar denaturation midpoints for hPAH and hTH1, this property is directly comparable for the two enzymes. Interestingly, the lower extent of aggregation observed for TH was similar to that of PAH in the presence of satu-

rating concentrations of phenylalanine [8]. Studies on chimeric proteins suggest that the N-terminal domain is important for the aggregation of PAH. In particular, exchange of the N-terminal domain of rat TH with that of rat PAH gave lower purification yield and higher amount of inclusion bodies than the wild type TH [24]. Furthermore, the N-terminal of TH was found to increase the solubility of PAH compared to the wild type [24]. Purification of the hPAH N-terminal domain has also proven to be difficult due to protein aggregation [25].

The process of protein denaturation depends on the stability of the native state relative to the transition and denatured states. Whereas the free energy of strongly unfolded proteins would be expected to be minimally affected by mutations, such simplification will not apply to more compact denatured states [26]. Hence, we expect that the partially denatured states of the AAAHs will play an important role in the stability of these enzymes, given the significant residual structure found for these states. Furthermore, since the partially denatured states are different between the hydroxylases, their response to homologous missense mutations may be less predictable than expected from studies on their native structures alone. It is tempting to speculate that the strong tendency of PAH to aggregate may explain the existence of numerous different mutations (>400) associated with phenylketonuria, as opposed to the paucity of known mutations in TH. The strong tendency of purified TPH to precipitate is puzzling and has prevented us from performing a complete comparison of all members of the AAAH family. Furthermore, the aggregating properties of the latter enzyme have raised the question whether TPH and the other AAAHs are stabilized in vivo by additional molecular interactions [10]. With such a low stability as reported here for hTH1 and hTPH1, one might speculate if it has a physiological significance in the regulation of catecholamine and serotonin biosynthesis, e.g., by placing more control of the biosynthesis to protein turnover.

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