Denatured states of human carbonic anhydrase II: an NMR study of hydrogen/deuterium exchange at tryptophan-indole-H_N sites

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Abstract Hydrogen/deuterium (H/D) exchange measurements in low and moderate concentrations of GuHCl were conducted on the side chain H_N atoms of the seven tryptophans of pseudo wild-type human carbonic anhydrase II. Tryptophans 5, 16 and 245, situated in or close to the N-terminal domain were found to have little protection against exchange. The H/D exchange results for Trp-123, Trp-192 and Trp-209 showed that a previously identified molten globule and the native state gave a similar protection against exchange. Global unfolding of the protein is necessary for the efficient exchange at Trp-97, which is located in the central part of the β-sheet.

Key words: Protein folding; Hydrogen/Deuterium exchange; NMR; Folding intermediate; Carbonic anhydrase; Molten globule

1. Introduction

Partly folded conformations are important constituents of the energy landscape of proteins. Characterization of the structure and stability of partly (un)folded protein molecules (intermediates) is therefore required for a complete understanding of the protein folding and structure. Partly folded conformations are also thought to be important in many other biological processes, such as protein translocation across membranes and formation of the amyloid fibers which are associated with several neurodegenerative diseases [1].

Various unfolded states of the 29 kDa enzyme human carbonic anhydrase II (HCAII) have been previously characterized by several investigators using different techniques. The global unfolding parameters of HCAII have been determined by studying the effects of denaturants using spectroscopic methods [2–5]. These investigations showed that HCAII unfolds in two transitions separated by a stable molten globule intermediate (I). These transitions will be referred to in the following text as the first and second unfolding transitions ($N \leftrightarrow I \leftrightarrow U$). In addition, the stability of local structures has been investigated using mutagenesis, which has also been combined with chemical labelling to generate various probes [5–8].

In near UV CD and fluorescence measurements we have also used the spectroscopic properties of the indole group in Trp to report on tertiary structure rearrangements in unfolded forms of HCAII [9–11]. These results have been combined with complementary data from studies of accessibility of engineered SH groups to iodoacetate, to map and characterize the compactness and stability of the unfolded states [5,6]. Near UV CD detects tertiary structures, since the spectrum is dependent upon immobilization of Trp and other aromatic residues in defined conformations in the tertiary structure. Intrinsic fluorescence is a sensitive indicator of the polarity of the environment and can therefore give information concerning the environment of Trp residues. In cases where the spectra from individual Trp residues can be recorded, one can obtain more detailed structural information, especially where the fluorescence from an individual Trp is quenched by a nearby histidine side chain when the protein adopts its native conformation [9,12].

In recent years a new technique has been developed, in which H/D exchange in combination with NMR is used to study proteins in native and mildly denaturing conditions [13–18]. In the present study we measured the H/D exchange that occurs in the Trp-indole groups of HCAII when the structure is perturbed by various concentrations of GuHCl. The H/D exchange approach is based on the observation that the rate of exchange between solvent and labile hydrogens (amide and Trp-indole) is dependent on their individual environments. In particular, hydrogens in native proteins that exchange at rates much slower than predicted from analysis of small peptides tend to be involved in intramolecular hydrogen bonds, or buried deeply within the protein core. This method has mostly been used to probe secondary structure (peptide-H_N) but when applied to Trp-indole-H_N analysis it can reveal compactness and hydrogen bonding further out from the backbone and thus indicate a tertiary structure. Notably, the H/D exchange method can generate data that are independent of the spectral properties of the Trp and which may, therefore, complement data from earlier results. The fluorescence from the Trp-indole is sensitive to alterations in the environment at every position in the ring system. In contrast, Trp-indole H/D exchange analysis is more specific and monitors only the solvent accessibility of the H_N in the ε position.

Recently, a complete backbone assignment of HCAII has been presented [19] and earlier we assigned the indole-H_N cross-peaks of Trp-5, 123, 192 and 209 from HSQC spectra of HCAII using ^{15}N labelling in combination with site-directed mutagenesis [9]. In the present study the remaining three indole-H_N cross-peaks of HCAII were assigned to the Trp-16, 97 and 245, which made it possible to monitor the H/D exchange at all Trp in low and moderate concentrations of GuHCl.

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Abbreviations: GuHCl, guanidine hydrochloride; $HCAII_{pwt}$, pseudo wild-type human carbonic anhydrase II; H/D exchange, hydrogen/deuterium exchange

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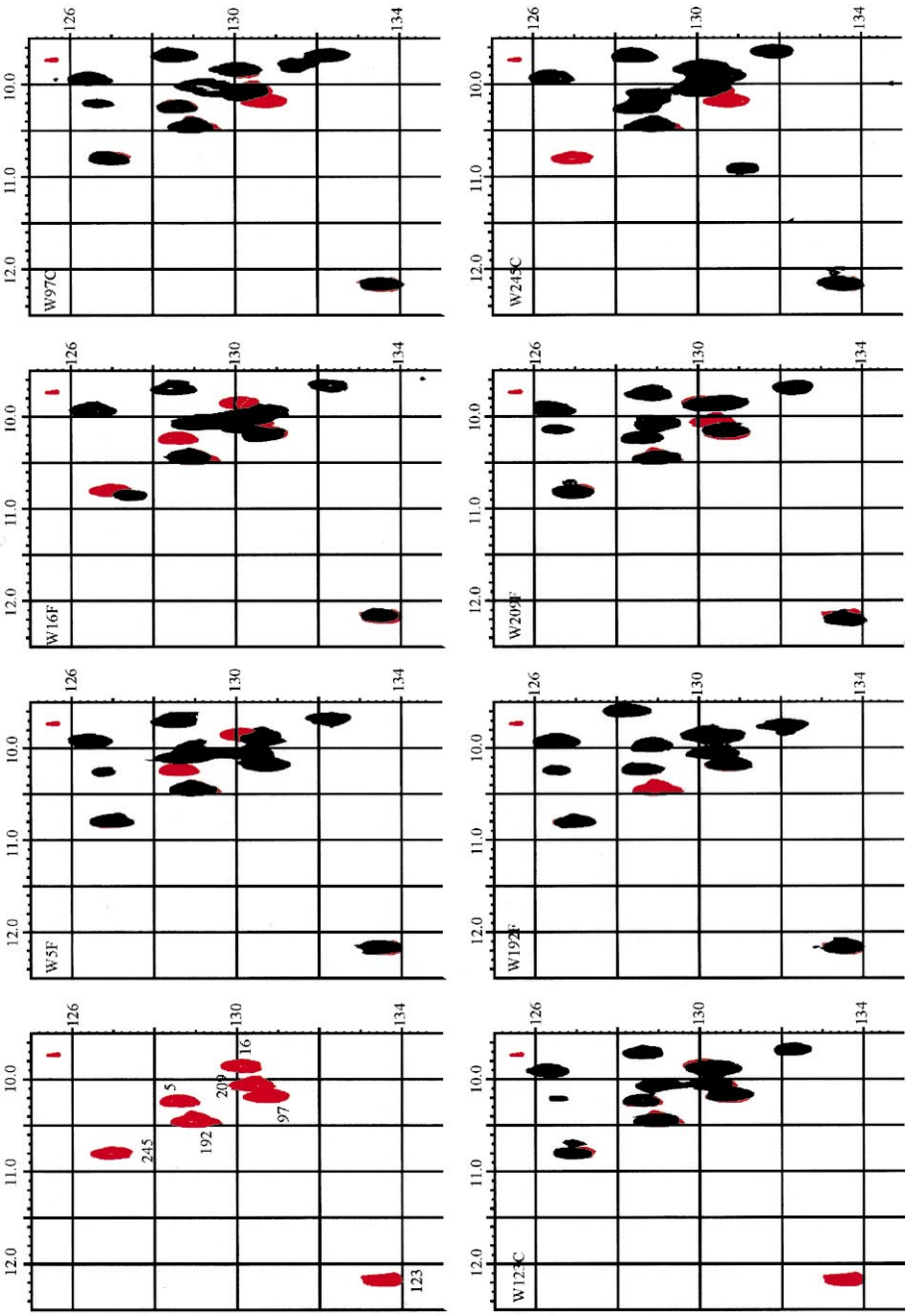

Fig. 1. ^{1}H-^{15}N HSQC spectra (in black) of the various tryptophan mutants of HCAII, superimposed on an HSQC spectrum (in red) of a sample with selectively ^{15}N-labelled tryptophans. The suggested assignment is indicated in the upper left spectrum of the selectively labelled sample.

2. Materials and methods

2.1. Chemicals

Isopropyl β-galactopyranoside (iptg) was obtained from saveen. Acrylamide from bdh was of electrophoresis grade, and all other chemicals were of reagent grade. D_2O (d 99.75%) was purchased from Merck and L-trp-$^{15}N_2$ (^{15}N 95-99%) from larodan fine chemicals.

2.2. Spectrometers

NMR measurements were performed on a Bruker AMX2 spectrometer operating at a proton frequency of 500 MHz. Absorbance and fluorescence measurements were performed on a Perkin-Elmer 320 spectrophotometer and a Shimadzu RF-5000 spectrofluorophotometer, respectively.

2.3. Protein production and purification

In vitro site-directed mutagenesis was performed as described in Mårtensson et al. [9]. All uniformly ^{15}N-labelled enzyme variants were produced and purified as described in Mårtensson et al. [9]. ^{15}N tryptophan-labelled pseudo wild-type human carbonic anhydrase II ($HCAII_{pwt}$) was produced using similar conditions to those described in Mårtensson et al. [9], except that the cells were grown in a defined medium consisting of 400 mg/l of Ala, Glu, Gln, Arg and Gly, 250 mg/l of Asp and Met, 100 mg/l of Asn, His, Ile, Lys, Thr, Tyr, Val and Leu, and 50 mg/l of Cys, cystine and Phe. The medium was supplemented with 125 mg/l of adenine, cytosine uracil and guanosine, 50 mg/l of thymine, 1 g/l NH_4Cl, 10 mg/l $CaCl_2$, 2 g/l NaAc, 10 g/l K_2HPO_4, 2 g/l of succinic acid, 0.5 mM $ZnSO_4$, and 50 mg/l ampicillin. The pH was adjusted to 7.4 with NaOH and 50 mg/l ^{15}N-labelled tryptophan was added before

inoculation, at the time of induction with IPTG, and 3 h after induction.

2.4. Equilibrium unfolding measurements

The GuHCl-induced global equilibrium unfolding was monitored by fluorescence and the data were analyzed as described in Mårtensson et al. [9]. The solvent was D_2O and the temperature was 30°C, at pD 7.2.

2.5. H/D exchange experiments

^{15}N-labelled $HCAII_{pwt}$ was dissolved in deuterated 0.2–0.9 M GuHCl, buffered with deuterated sodium-phosphate buffer, pD 7.2, to a concentration of 1 mM. The H/D exchange rates were monitored by 2D HSQC NMR (heteronuclear single quantum coherence) in the sensitivity-enhanced gradient version as described by Kay et al. [20]. Square gradients of 1 ms duration were applied with strengths of 24 and 3 G/cm for the 1st and 2nd gradients respectively. The spectra were recorded with an acquisition time of 20 min upon solvation of the protonated protein. In the 0–120 minutes time span, data were recorded continuously. Thereafter, data were collected 3 h, 6 h, 12 h, 1 day and 2–5 days after the initial exposure to D_2O. The exchange data were fitted to a single exponential decay function using the program Grafit (Erithacus Software Ltd, Staines, UK).

2.6. Theory

Hydrogens involved in intramolecular hydrogen bonds or shielded by a compact structure are protected from exchange with solvent deuterons [15,21]. To allow exchange, these structures must be opened to the solvent in an opening/closing reaction [13]

$$HX_{closed} \leftrightarrow^{k_{op}}_{k_{cl}} HX_{open} \xrightarrow{k_{rc}} DX$$

$$K_{op} = k_{op}/k_{cl}$$

The exchange rate for a given hydrogen is defined as [15]

$$k_{ex} = k_{op}k_{rc}/(k_{op} + k_{cl} + k_{rc})$$

At the so called EX2 limit ($k_{cl} \gg k_{rc}$), which holds for most amide and side chain hydrogens in proteins [17] and when the native conformation is predominant ($k_{op} < k_{cl}$), the exchange rate expression can be modified to

$$k_{ex} = (k_{op}/k_{cl})k_{rc} = K_{op}$$

From this relationship the free energy (ΔG_{op}) for the opening reaction can be calculated according to

$$\Delta G_{op} = -RT\ln K_{op} = -RT\ln(k_{ex}/k_{rc})$$

where k_{ex} is the measured exchange rate and k_{rc} is the H/D exchange rate for H_N in observed model peptides [22]. The values of k_{rc} for the Trp-indole-H_N are taken from Bai et al. [22] without any correction for effects on the exchange by nearest neighbor side chains. Steric hindrance and inductive effects from neighboring side chains on exchange at indole-H_N in random chains have not yet been measured, to our knowledge, but are likely to be small.

The dependence of ΔG_{op} on denaturant concentration is often expressed as

$$\Delta G_{op} = \Delta G_{H_2O} - m[\text{denaturant}]$$

.where m is a constant reflecting the newly exposed denaturant binding sites upon unfolding [23,24]. The ΔG_{op} and m values for different hydrogens obtained in a plot of ΔG_{op} versus [denaturant] can be usefully compared with corresponding values for the global unfolding transition obtained in equilibrium unfolding experiments monitored by spectroscopic techniques. Structural components that unfold according to local or global unfolding mechanisms can then be identified.

3. Results and discussion

Four of the indole-H_N cross-peaks in the HSQC spectrum of HCAII have previously been assigned by Mårtensson et al. [9]. The assignment has now been completed, with the results presented here, using specific ^{15}N labelling of Trp in combination with site-directed mutagenesis to substitute the Trp with other amino acid residues. The locations of the different Trp-indole-H_N in the 2D NMR spectrum are shown in Fig. 1 and their positions in the protein are shown in Fig. 2.

As can be seen in Fig. 1, assignment of all the Trp-indole-H_N apart from Trp-5 and 16 is quite straightforward and careful inspection of the spectra suggests that the most likely assignment of these Trp is the one proposed.

The equilibrium unfolding of HCAII, which has been extensively studied with various methods, has been found to occur according to a three state model including a partly denatured state (molten globule intermediate) around 1.5 M GuHCl. The investigations have shown that the molten globule retains much of the secondary structure. To complement these data we decided to characterize the native and partly unfolded states of HCAII in detail using H/D exchange experiments.

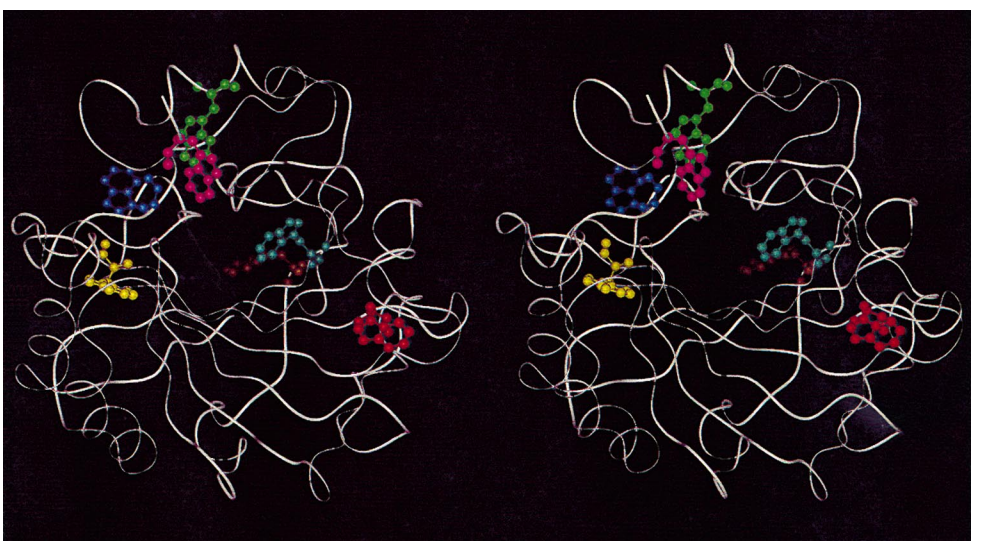

Fig. 2. A stereo representation of the HCAII backbone and the tryptophan side chains: Trp-5 (pink), Trp-16 (green), Trp-97 (yellow), Trp-123 (red), Trp-192 (brown), Trp-209 (turquoise) and Trp-245 (blue).

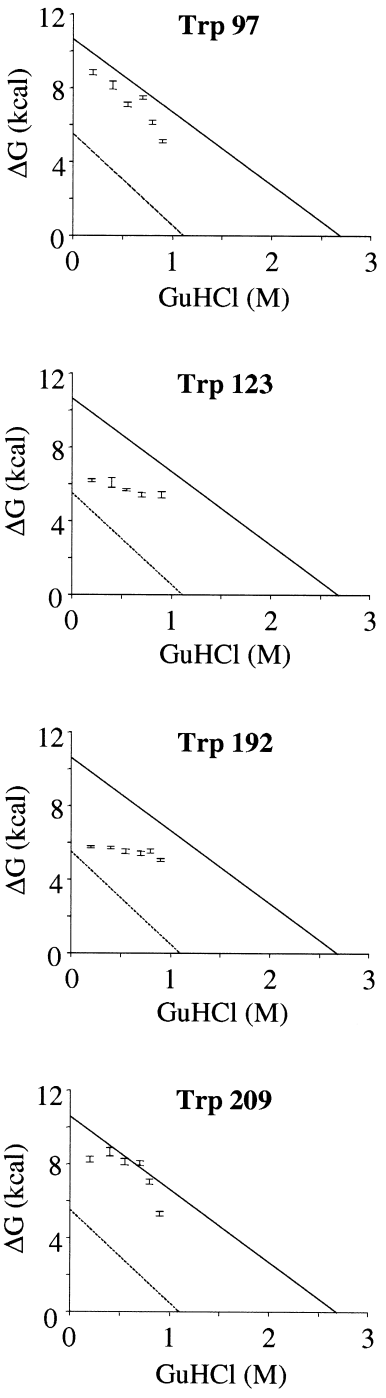

Fig. 3. The ΔG_{op} values (I) from the H/D exchange of Trp-indole-H_N of $HCAII_{pwt}$ as a function of the GuHCl concentration. The ΔG values for GuHCl induced unfolding monitored by Trp fluorescence are represented by the N→I transition (dotted line) and the I→U transition (solid line).

3.1. Residues exchanging through global unfolding

Trp-97 is situated within the central part of the β-sheet (β-strand 4) and is a component of the large hydrophobic cluster that is situated below the central β-strands in the view presented in Fig. 2. The indole-H_N of Trp-97 is involved in a hydrogen bond to the main chain oxygen atom of Met-241 (H-O distance 2.06 Å, [25]). It exchanges through a global unfolding mechanism, the I↔U transition (Fig. 3), and shows the largest protection factor against H/D exchange of all tryptophans in HCAII. Earlier investigations, using a $HCAII_{pwt}$ variant with a cysteine in position 97 and chemical labelling techniques, have shown that the γ atom of position 97 is involved in a residual structure which is protected against solvent up to 4 M GuHCl. This structure is not totally ruptured until strong denaturing conditions (>8 M GuHCl) are applied [5,6]. The near UV CD spectrum of native HCAII is dominated by bands from Trp-indoles and their individual contributions to the spectrum have been determined [10]. Notably, all of these bands disappear in the first transition (N↔I) when denatured by GuHCl, which indicates that the Trp-indoles become mobile in the molten globule, i.e. they are no longer confined in asymmetric environments.

3.2. Residues exchanging through local unfolding mechanisms

At low denaturant concentrations Trp-123, 192 and 209 exchange through local unfolding. This exposes little or no additional denaturant binding surface ($m=0$) and the exchange is unaffected by the first transition, N↔I. Trp-123 and 192 are situated in β-strand 5 and 8, respectively. The indole-NH of Trp-123 is hydrogen bonded to the side chain carboxyl oxygen of Asp-72 (H-O distance 1.91 Å) and the Trp-192 indole-H_N is hydrogen bonded to a 'buried' H_2O molecule (H-O distance 2.04 Å). Trp-209 is situated in β-strand 7 and is hydrogen bonded to the Ser-197 carbonyl oxygen (H-O distance 2.07 Å). The local stability at Trp-209 is high (Fig. 3) and therefore the exchange at high GuHCl concentrations becomes dominated by the global unfolding, hence it seems to follow the global I↔U transition above 0.8 M GuHCl. Measurements of accessibility of the SH group to iodoacetate in a cysteine variant of Trp-123 have shown that the γ position in this residue is situated in a compact environment in the molten globule [5]. They have also shown that the γ position becomes accessible to the solvent in the second transition, I↔U. The fluorescence of each Trp shifts towards longer wavelengths upon denaturation. However, in the molten globule, the fluorescence spectra of Trp-97, Trp-123, Trp-192 and Trp-209 has shifted approximately half way towards their shifts in the unfolded state, indicating that they are situated in compact and rather apolar environments [9]. The fluorescence spectrum of Trp-5 is fully red-shifted in the molten globule and identical to the spectrum in the unfolded state.

3.3. Rapidly exchanging residues

The indole-H_N groups of Trp-5, 16 and 245 exchange in the dead time of the experiment (20 min) even at the lowest concentrations of GuHCl, i.e. they have very low protection factors. The indole-NH of Trp-5 projects from the protein surface and has no protein hydrogen bonding partner, while the indole-NH of Trp-16 seems to have a hydrogen bond to the backbone carbonyl oxygen of Gly-6 (H-O distance 2.23 Å).

Earlier kinetic investigations of the folding process have shown that the N-terminal 24 amino acid residue segment of HCAII (which is not needed for the enzyme to be active) folds onto the rest of the molecule in the final folding step [26].

Trp-245 is located in a loop connecting the C-terminal β-strand 9 with a helical segment (residues 220–226) and its indole-H_N is hydrogen bonded to a carboxyl oxygen of Asp-243 (H-O distance 2.03 Å). Earlier investigations monitoring accessibility to chemical labelling at a cysteine inserted in position 245 have shown that the SH-group extends towards

the stable central part of the β-sheet and is not exposed in the first transition but becomes accessible in the second transition I↔U [6]. A high flexibility of the N-terminal segment would allow a rapid H/D exchange at those indole-H_N.

4. Conclusions

To get a comprehensive picture of the folding process from the analysis of H/D exchange at Trp residues, it is necessary to compare them to earlier results from measurements of CD, fluorescence and solvent accessibility at engineered cysteine residues. It is clear that, in probing the Trp environment, each of these methods will yield distinct information. Trp fluorescence is sensitive to changes in polarity at all positions around the indole group (i.e. it is impossible to differentiate between changes that occur around different parts of the indole). The near UV CD spectrum is dependent on the immobilization of aromatic residues (especially Trp) in defined conformations in the tertiary structure. Thus, diminishment of near UV CD bands report an increased mobility of the Trp-indole. Cys residues, introduced by mutagenesis, can be used to probe compactness at the side chain β position with a high precision since the exposure of the side chain to the solvent can be inferred from measurements of the rate of alkylation by iodoacetate. The results concerning H/D exchange at indole-H_N sites give information on events at the ε position that allow the NH group contact with D_2O for sufficient time to allow exchange to occur. Hydrogen bonds between the indole-H_N and other groups in the protein must be broken and the proton and its acceptor atom must be displaced by at least 3 Å for efficient H/D exchange to take place [18]. This is because the exchange reaction involves the transient formation of a new hydrogen bond to an OH^- ion from the solvent.

The first unfolding transition, N↔I, has a midpoint at 1.2 M GuHCl and is indicated by a complete loss of enzymic activity, disappearance of all near UV CD bands and a pronounced red shift of the fluorescence spectrum. Data from far UV CD measurements indicate, however, that the major part of the secondary structure remains intact in the molten globule (I). Thus many of the well-defined interactions that the Trp-indoles have in the native state appear to be lost in this transition, which must involve an increased mobility of all Trp-indoles as well as more frequent interactions between the Trp-indoles and bulk water. However, the N↔I transition (dotted line in Fig. 3) does not correlate to the H/D exchange at the indole-H_N of Trp-97, Trp-123, Trp-192 and Trp-209. But there is a clear correlation between the extrapolated ΔG values for the I↔U transition (solid line in Fig. 3, measured by Trp fluorescence) and the ΔG_{op} values from H/D exchange for Trp-97 and partly for Trp-209. Notably, Trp-97 and Trp-209 are the most deeply buried Trp residues in HCAII.

Taken together, these data indicate that major features of the molten globule (I) of HCAII include a preserved secondary structure, the loss of specific side chain interactions, an increased mobility of Trp side chains and more frequent contact with the bulk water than in the native state for the outer parts of the side chains of Trp-97, Trp-123, Trp-192 and Trp-209. However, the H/D exchange results show that the fluctuations at these Trp residues do not allow H_2O to penetrate the molten globule deeply enough to reach the ε position of the Trp side chains, i.e. the molten globule seems to be as dry as the native state. A recent ^{17}O NMRD study by Denisov et al. [27] of the native and molten globular states of HCAII and other proteins has shown that the molten globules of these proteins retain most native internal water molecules, but they are not otherwise significantly penetrated by solvent.

The H/D exchange results (ΔG_{op} nearly constant up to 0.8 M GuHCl, Fig. 3) show that the local unfolding that govern the exchange at Trp-123, Trp-192 and Trp-209 seems to be essentially unaffected by the increase of the mobility of the side chains that occur in the N↔I transition (dotted line in Fig. 3). Hence, the local structures around Trp-123, Trp-192 and Trp-209 seem to be stabilized by general hydrophobic interactions, possibly by transient hydrogen bonding at the indole NH and by backbone hydrogen bonding interactions, which might remain essentially native in the molten globule.

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References

[1] Kelly, J.W. (1996) Curr. Opin. Struct. Biol. 6, 11–17.
[2] Carlsson, U., Henderson, L.E. and Lindskog, S. (1973) Biochim. Biophys. Acta 310, 376–387.
[3] McCoy, L.F., Rowe, E.S. and Wong, K.-P. (1980) Biochemistry 19, 4738–4743.
[4] Henkens, R.W., Kitchell, B.B., Lottich, C.S., Stein, P.J. and Williams, T.J. (1982) Biochemistry 21, 5918–5923.
[5] Mårtensson, L.-G., Jonsson, B.-H., Freskgård, P.-O., Kihlgren, A., Svensson, M. and Carlsson, U. (1993) Biochemistry 32, 224–231.
[6] Svensson, M. et al. (1995) Biochemistry 34, 8606–8620.
[7] Lindgren, M., Svensson, M., Freskgård, P.-O., Carlsson, U., Jonasson, P., Mårtensson, L.-G. and Jonsson, B.-H. (1995) Biophys. J. 69, 202–213.
[8] Hammarström, P., Kalman, B., Jonsson, B.-H. and Carlsson, U. (1997) FEBS Lett. 420, 63–68.
[9] Mårtensson, L.-G., Jonasson, P., Jonsson, B.-H., Freskgård, P.-O., Svensson, M. and Carlsson, U. (1995) Biochemistry 34, 1011–1021.
[10] Freskgård, P.-O., Mårtensson, L.-G., Jonasson, P., Jonsson, B.-H. and Carlsson, U. (1994) Biochemistry 33, 14281–14288.
[11] Andersson, D., Freskgård, P.-O., Jonsson, B.-H. and Carlsson, U. (1997) Biochemistry 36, 4623–4630.
[12] Loewenthal, R., Sancho, J. and Fersht, A.R. (1991) Biochemistry 30, 6775–6779.
[13] Englander, S.W., Englander, J.J., Mcinnie, R.E., Ackers, G.K., Turner, G.J., Westrick, J.A. and Gill, S.J. (1992) Science 256, 1684–1687.
[14] Mayo, S.L. and Baldwin, R.L. (1993) Science 262, 873–876.
[15] Bai, Y., Milne, J.S., Mayne, L. and Englander, S.W. (1994) Proteins 20, 4–14.
[16] Bai, Y., Sosnick, T.R., Mayne, L. and Englander, S.W. (1995) Science 269, 192–197.
[17] Bai, Y. and Englander, S.W. (1996) Proteins 24, 145–151.
[18] Milne, J.S., Mayne, L., Roder, H., Wand, A.J. and Englander, S.W. (1998) Protein Sci. 7, 739–745.
[19] Venters, R.A., Farmer, B.T., Fierke, C.A. and Spicer, L.D. (1996) J. Mol. Biol. 264, 1101–1116.
[20] Kay, L.E. (1993) J. Am. Chem. Soc. 115, 2055–2057.
[21] Hvidt, A. and Nielsen, S.O. (1966) Adv. Protein Chem. 21, 287–386.
[22] Bai, Y., Milne, J.S., Mayne, L. and Englander, S.W. (1993) Proteins 17, 75–86.
[23] Pace, C.N. (1975) CRC Crit. Rev. Biochem. 3, 1–43.
[24] Schellman, J.A. (1978) Biopolymers 17, 1305–1322.
[25] Eriksson, E.A., Jones, T.A. and Liljas, A. (1988) Proteins 4, 274–282.
[26] Aronsson, G., Mårtensson, L.-G., Carlsson, U. and Jonsson, B.-H. (1995) Biochemistry 34, 2153–2162.
[27] Denisov, V.P., Jonsson, B.-H. and Halle, B. (1998) Nat. Struct. Biol. (in press)