

Pyrene excimer fluorescence as a proximity probe for investigation of residual structure in the unfolded state of human carbonic anhydrase II

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Abstract The excimer fluorescence from two pyrenyl moieties attached to cysteines in human carbonic anhydrase II has been monitored to characterize residual structure retained under strong denaturing conditions. A position in β -strand 3, N67C, together with the single naturally occurring cysteine 206 in β -strand 7, were used as attachment sites. The excimer formation by the pyrenyls, requiring proximity of the probes, revealed an unfolding transition at a GuHCl concentration significantly higher than that required to induce unfolding of the molten globule state as monitored by CD. These results indicate that the excimer transition monitors the unfolding of a residual compact structure that spans β -strands 3–7. This region constitutes the central and the most hydrophobic part of the molecule, emphasizing the importance of hydrophobic interaction in maintaining residual structure under strong unfolding conditions.

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Key words: Excimer fluorescence; Pyrene; Folding; Carbonic anhydrase; Residual structure

1. Introduction

We are using the enzyme human carbonic anhydrase II (HCAII) as a model protein in our investigations of the protein folding mechanism [1]. The crystal structure of HCAII has been determined at high resolution [2,3]. The active site contains a zinc ion coordinated to three histidine residues. The structure is illustrated schematically in Fig. 1. The overall shape of the protein is ellipsoidal with the dimensions $39 \times 42 \times 55$ Å, and the molecular mass is 29 kDa. HCAII consists of 10 β -strands connected by hairpin loops and some helices. The open β -sheet stretches throughout the entire molecule and divides it into two halves: the upper half includes the N-terminal helical region and the active site, and the lower half contains a large hydrophobic core. This core has previously been shown to be resistant to unfolding, even at high concentrations of GuHCl [4,5].

Under moderate denaturing conditions, HCAII and mutants thereof demonstrate characteristic features of a molten globule state. The rate of alkylation of engineered cysteines has revealed that some sulfhydryl groups are remarkably inaccessible to alkylation, even at high (> 5 M) GuHCl concentrations [4,5]. This indicates that part of the protein structure,

i.e. β -strands 3–5, remains as a rather compact structure even under strongly denaturing conditions. Residual structures have also been detected in other proteins, that were previously reported to be completely unfolded when examined by conventional optical methods. By employing more specific techniques to study the unfolding process, for example NMR, residual structure has been shown to persist in several proteins [6–10]. Residual structure in the unfolded state has been reviewed [11], and has also been suggested to act as ‘seeds’ that initiate the folding reaction [12]. Therefore, it is of great interest to characterize such structures. We have initiated a series of studies to map the topology and boundaries of residual structures that exist at high denaturant concentrations. Obviously, it is of the utmost importance that a broad repertoire of methods be developed that can give complementary structural information. For this purpose, we introduce the use of pyrene excimer fluorescence measurements in combination with site-directed mutagenesis.

If two pyrenyl groups are close to each other (i.e. within a few Å), they can form an excited state dimer (excimer) upon excitation [13]. Compared to excited monomers, excimers produce a red-shifted spectrum. Consequently, excimer formation is a versatile tool for probing rather short distances between amino acid residues, and it gives information that complements energy transfer measurements, in which the probed distances should, ideally, agree with the Förster radius (frequently 30–100 Å).

In the experiments described here, we specifically attached pyrenyl fluorophores as proximity probes to a pair of cysteine residues. The steric interference of the relatively bulky pyrenyl moieties with the protein is of less significance in the unfolded or partially folded states that we study in this report than in the more compact native state. This method is therefore suitable for studies of conformational changes in the unfolded state. Pyrene excimer fluorescence has previously been used to study unfolding of the native state of tropomyosin [14] and the distance between membrane-spanning helices [15].

2. Materials and methods

2.1. Materials

N-(1-pyrenemethyl)iodoacetamide, PMIA, was obtained from Molecular Probes. Sequential grade GuHCl was purchased from Pierce and pure grade GuHCl was obtained from Merck. The GuHCl was treated as described previously [4] and the concentrations were determined by refractive index [16].

2.2. Spectroscopic measurements

Fluorescence spectra were recorded on a Hitachi F-4500 fluorometer equipped with a thermostated cell. The measurements were conducted in 1 cm quartz cells at 23°C with an excitation wavelength of 344 nm. Absorbance spectra were obtained with a Hitachi U-2000 spectrophotometer in 1 cm quartz cells. CD spectra were recorded

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Abbreviations: DMSO, dimethylsulfoxide; GuHCl, guanidine hydrochloride; HCAII, human carbonic anhydrase II; PMIA, *N*-(1-pyrenemethyl)iodoacetamide; py-N67C/C206-py, pyrene-labeled cysteines in a HCAII mutant with a Asn-67 \rightarrow Cys-67 substitution

on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA), employing constant N_2 flushing.

2.3. Molecular modeling

Simulations of the mutation in HCAII were performed using the computer program HyperChem (Hypercube Inc.). Molecular modeling of the N67C mutation was performed using the OPLS force field [17] and a Polak Ribiere algorithm with 0.1 kcal/(Å mol) as termination condition.

2.4. Production of mutated protein

Site-directed mutagenesis (N67C/C206), protein production and purification was performed as described previously [4,18,19]. C206 is the unique cysteine in HCAII and is highlighted in this paper because it is used as one of the attachment sites for a pyrene probe. Protein concentrations were determined from absorbance at 280 nm assuming the extinction coefficient of the mutant to be equivalent to the wild-type protein $\epsilon_{280} = 54\,800\text{ M}^{-1}\text{ cm}^{-1}$. Enzyme activity (CO_2 hydration) was measured as described elsewhere [20,21].

2.5. Stability measurements

The protein stability was determined using far-UV CD and fluorescence measurements. The protein was incubated prior to the measurements for 24 h at 23°C in various concentrations of GuHCl buffered with 0.1 M Tris- H_2SO_4 , pH 7.5. Protein concentrations were 8.5 μM (far-UV CD) and 0.85 μM (fluorescence).

2.6. Pyrene labeling

The N67C/C206 mutant was labeled in the unfolded state with PMIA. Fifteen mg of protein was dissolved in 7.0 M GuHCl in 0.1 M Tris- H_2SO_4 , pH 8.5 buffer and to prevent formation of disulfide bridges a 2-fold molar excess of β -mercaptoethanol over protein was added. 4.1 mg of PMIA was dissolved in 100 μl of DMSO, and was added in aliquots during mixing. The reaction mixture was left for 24 h at 37°C in the dark on a mechanical shaker. The reaction was quenched by addition of 100 μl of 0.2 M β -mercaptoethanol (2-fold molar excess over PMIA) and centrifuged to remove precipitated reagent. The protein was refolded by dilution to 0.10 M GuHCl in 0.1 M Tris- H_2SO_4 , pH 7.5, with a protein concentration of 0.03 mg/ml. The labeled protein was concentrated with Amicon YM-10 filter and in a Diaflo centrprep 10 (1100 $\times g$, 30 min) to 2 ml. The turbid solution was filtered and gel filtrated. The gel filtration was performed on a Sephadex G-25 column (coarse gel; Pharmacia) equilibrated with 10 mM Na-borate buffer, pH 7.5.

2.7. Determination of the degree of labeling

The concentration of the PMIA adduct was determined spectrophotometrically, using $\epsilon_{344} = 41\,000\text{ M}^{-1}\text{ cm}^{-1}$ [22]. The protein concentration was determined at 280 nm after subtraction of the absorbance of the probe.

3. Results

HCAII has one naturally occurring cysteine residue, C206, which is located in β -strand 7. C206 was used as one of the attachment sites for the pyrene moiety and the engineered N67C, in β -strand 3, as the other attachment site. Computer modeling showed an angle between the side chains C67 and C206 ($C\beta$ -S) of 60° and a sulfur-sulfur distance of 15 Å, which indicated that attachments to these sites would be favorable for formation of excimers.

3.1. Characterization of the N67C/C206 mutant

A specific enzymatic activity of 88% for the N67C/C206 mutant (CO_2 hydration activity) was retained as compared to the wild-type enzyme. Since the activity of an enzyme is very sensitive to structural alterations, and because the activity of the studied mutant was almost intact, it is not likely that the mutation caused any major conformational changes. Furthermore, the midpoint concentrations of denaturation are very similar to the wild-type HCAII (0.9 M and 2.3 M GuHCl (data not shown) for the native \rightarrow molten globule state and molten globule \rightarrow unfolded state transition, respectively).

3.2. Incorporation of label

During the labeling procedure, a high concentration of GuHCl was used due to the difficulty of gaining access to the cysteines 67, and 206 [5]. The degree of labeling of the N67C/C206 mutant was found to be 1.8–2.0 pyrenes per protein molecule in 3 different labeling experiments. 10% of the enzymatic activity was regained when py-N67C/C206-py was subjected to conditions favoring the native state. This recov-

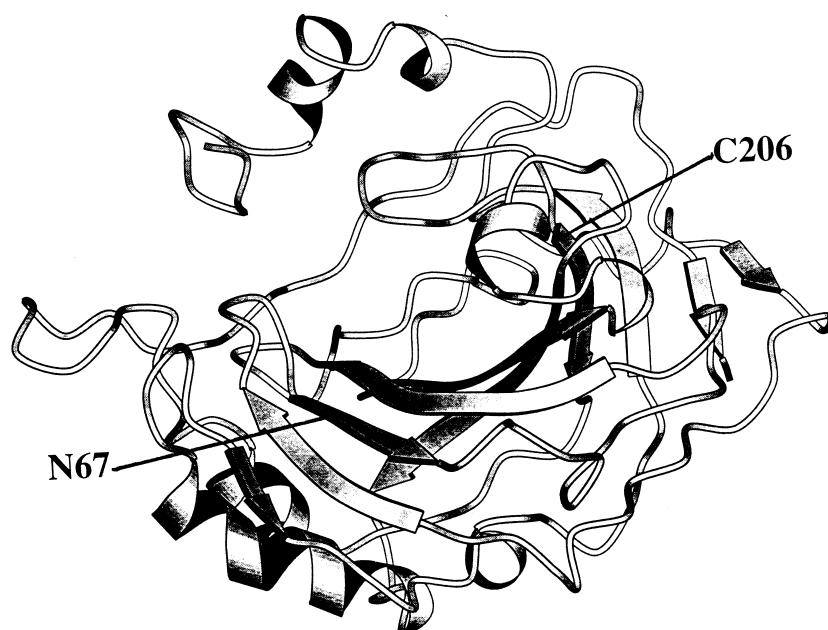


Fig. 1. Schematic view of the polypeptide backbone of human carbonic anhydrase II with the mutated and labeled sites indicated. Picture drawn using the program MOLSCRIPT [32].

ery of activity could possibly be due to a small fraction of unlabeled enzyme. The low degree of reactivation of the modified enzyme is not unexpected, since interference with the formation of the vicinal active site is likely to occur. Since we are primarily interested in the conformations of residual structures that are present under strong denaturing conditions in the unfolded state, perturbation of the native state is of less significance.

3.3. CD measurements

The near-UV CD spectrum in Fig. 2b shows py-N67C/C206-py and as a comparison the spectrum of HCAII is also included. This CD spectrum of py-N67C/C206-py has clear interferences from the pyrene moieties with distinct peaks at 247 nm and 278 nm, but is otherwise similar to the HCAII near-UV spectrum. This indicates that py-N67C/C206-py has folded into a native-like tertiary structure [18]. CD measurements in the far-UV wavelength region, Fig. 2a, display a different spectrum than that of the unlabeled HCAII, presumably due to contribution from the pyrenyl moieties. Nevertheless, changes in the CD signal at 222 nm were considered to reflect changes in secondary structure. After incubation of py-N67C/C206-py in various concentrations of GuHCl, the mean residue ellipticity at 222 nm was

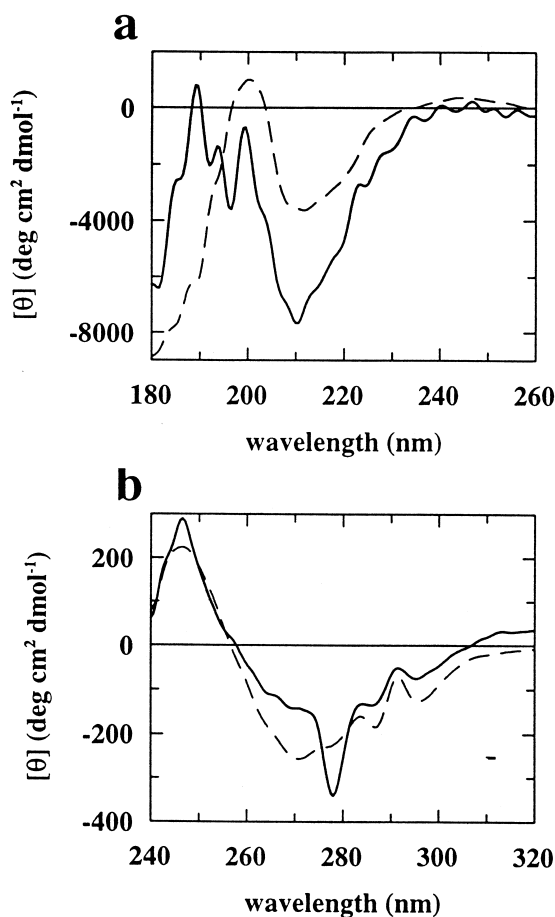


Fig. 2. CD spectra of py-N67C/C206-py (—) and HCAII (---). The mean residue ellipticity was measured. a: Far-UV spectra recorded in a 0.1 mm cuvette with a protein concentration of 17 μ M in 10 mM Na-borate buffer pH 7.5. b: Near-UV spectra recorded in a 0.5 cm cuvette with conditions as in a.

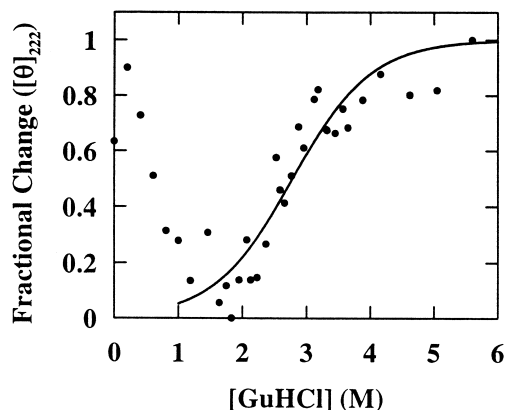


Fig. 3. Unfolding of py-N67C/C206-py monitored by change in mean residue ellipticity at 222 nm. Protein concentration was 8.5 μ M in varying concentrations of GuHCl and 0.1 M Tris- H_2SO_4 , pH 7.5 in a 0.5 mm cuvette.

monitored and plotted versus GuHCl concentration in Fig. 3. Between 0–1 M GuHCl a decrease in ellipticity was recorded, due to loss of contribution from tryptophan residues [18], when transition to the molten globule state occurs ([4], Kristina Borén, personal communication). Thus, the native state appears to be somewhat destabilized because of steric interferences from the pyrene groups (Fig. 3). In the interval 1.7–3.5 M GuHCl an increase was detected. The second CD transition is indicative of loss of secondary structure. Curve fitting was performed between 1.2–5.5 M GuHCl, for the data in Fig. 3. The midpoint concentration of transition was at 2.8 M GuHCl, which is at higher GuHCl concentrations than the corresponding transition of the unlabeled protein (2.3 M) which was monitored by Trp fluorescence. This could indicate that the molten globule state is stabilized by the pyrene moieties. The stabilization is probably not due to strong interactions between the two pyrene probes (see Section 3.5), but it is possible that the attached hydrophobic pyrene groups are located in the protein interior and thus contribute to the stability of the molten globule state. A similar effect was previously observed for a L118C substitution in HCAII [4], where the removal of a hydrophobic leucine in the protein core destabilized the molten globule state. Another possibility is that the observed difference could be due to spectroscopic interference from the pyrene moieties.

3.4. Fluorescence emission spectra

Fig. 4a shows the fluorescence emission spectrum of pyrenyl-labeled β -mercaptoethanol (py- β -merc). The spectrum is typical of a monomeric pyrenyl. Fig. 4b shows the fluorescence spectra of py-N67C/C206-py in presence and absence of GuHCl. The spectra exhibit a broad structureless band centered at 460–480 nm at low and intermediate concentrations of GuHCl. This band is diagnostic for an excimer formed from pyrenyl probes [23]. The excimer emission peak is around 465 nm, which shows that the probes are in an apolar environment [24]. The excimer band disappears at high concentrations of GuHCl.

3.5. Excitation spectra

Excitation spectra were recorded to investigate whether ground state interactions occurred between the pyrenyl probes. Excitation spectra were recorded at 280–360 nm for

py-N67C/C206-py. The pyrene emission was monitored at both 379 and 460 nm to detect the fluorescence from excited monomeric pyrene and that from excimers [25]. The two excitation spectra differ, and the peaks of the spectra detected at 460 nm, i.e. excimer fluorescence, are red-shifted and slightly broadened, as compared to the peaks of the spectra detected at 379 nm, i.e. monomer fluorescence (data not shown). The difference between the spectra indicates that pyrene moieties that form excimers had already formed dimers prior to excitation, or that these moieties were in a different environment than the non-excimer-forming pyrenyl moieties were. This is a ubiquitous feature of the formation of excimers from pyrenyls attached to proteins in aqueous solution [23]. Studies of the time dependence of the excimer fluorescence have shown that there is no build-up phase verifying that the excimers are formed from ground state complexes of pyrene [26]. Despite the possibility of ground state interaction between the probes, it is highly probable that the interaction is weak. Different pyrenyl-labeled proteins vary greatly in regard to the GuHCl concentration at which excimer fluorescence disappears [14,27], which strongly implies that the dimerization of pyrene in the ground state has no major effect on protein denaturation. However, the introduction of hydrophobic groups like pyrenes can affect the stability of a protein as discussed above.

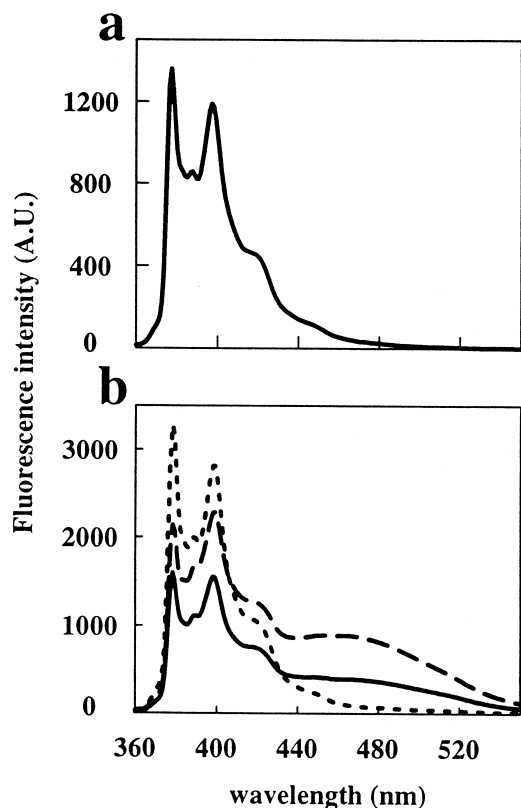


Fig. 4. Selected pyrenyl fluorescence emission spectra. a: Emission from pyrenyl-labeled β -mercaptoethanol (2 μ M) is shown. b: Emission from py-N67C/C206-py in 0.0 M (—), 3.0 M (---) and 5.1 M (-.-) GuHCl. Excitation was performed at 344 nm with 5 nm excitation and 2.5 nm emission slits in all measurements. Protein concentrations were 2 μ M and buffered by 0.1 M Tris- H_2SO_4 , pH 7.5.

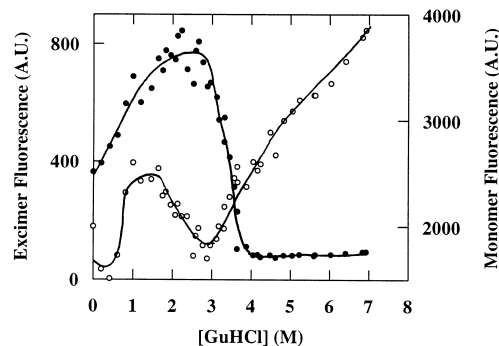


Fig. 5. Unfolding transition curves for py-N67C/C206-py as monitored by pyrene fluorescence. Monomer fluorescence intensity (\circ) recorded at 379 nm and excimer fluorescence intensity (\bullet) recorded at 465 nm. (Lines drawn to guide the eye.)

3.6. GuHCl-induced unfolding monitored by pyrene excimer fluorescence

The fluorescence from pyrene excimers was monitored in the range 440–550 nm. A transition curve was obtained for py-N67C/C206-py after incubation in various concentrations of GuHCl by plotting the excimer emission intensity versus GuHCl concentration (Fig. 5). Essentially an identical transition curve was obtained when the protein was unfolded in 6 M GuHCl and then diluted to lower GuHCl concentrations, showing that the unfolding (folding) reaction is reversible in the GuHCl concentration range 2.5–6 M.

3.7. GuHCl-induced unfolding monitored by pyrene monomer fluorescence

The pyrene monomer fluorescence was also monitored (Fig. 5). The monomer fluorescence continues to increase at concentrations of GuHCl above 4 M, where the excimer fluorescence has disappeared. Since the pyrene monomer fluorescence intensity (at 379 nm) of a pyrene β -mercaptoethanol adduct is independent of GuHCl concentration (data not shown) the increase in monomer fluorescence is an inherent feature for the py-N67C/C206-py molecule. This observed variation indicates a change in the environment surrounding the probes. It is likely that the monomer fluorescence increases as the excimers are separated, i.e. the concentration of the monomers increases, and it might also reflect loss of quenching from charged residues in the folded state of the protein. This could indicate that the pyrene moieties are quenched in 4 M GuHCl but as the protein is further unfolded in the interval 4–7 M GuHCl this quenching decreases. In a complementary experiment py-N67C/C206-py was fragmented with trypsin. Several Lys and Arg residues, where trypsin cleaves the polypeptide, are present in the sequence. One sample was pretreated with trypsin for 1 h and then solvated in 4.5 M GuHCl, and another sample was solvated in 4.5 M GuHCl without protease treatment. The trypsin-treated sample had 30% higher monomer fluorescence intensity than the uncleaved sample. This indicates that the environment quenching the pyrene fluorescence is partly destroyed. The quenching might originate from closely located charged residues.

4. Discussion

Under strong denaturing conditions we have earlier ob-

served residual structure in HCAII that persists after the molten globule \rightarrow unfolded state transition [4,5]. In the present study we have further explored the borders of this residual structure by labeling β -strands 3 and 7 of the protein with two pyrenyl moieties, which are used as proximity probes through their ability to form excimers.

Excimer formation has distinct geometric requirements and is therefore a versatile tool for studying unfolding. As long as two pyrenyl moieties, attached to a protein, are within a few Å distance an excimer band can be seen in the fluorescence spectrum. Extensive unfolding of the protein would separate the sites and the band would disappear. In order to prevent excimer formation due to local interactions we chose attachment sites which had close proximity in the native state and were well separated in the primary sequence.

Py-N67C/C206-py displayed excimer fluorescence in the folded state, and raising the concentration of GuHCl made the excimer fluorescence increase in the interval 0–1.7 M GuHCl with a plateau between 1.7–3 M GuHCl followed by a sharp decrease above 3 M. The band had virtually disappeared at 4 M GuHCl. Thus, the favorable excimer interaction that is observed between 1.7 and 3 M GuHCl (Fig. 5) indicates that a compact structure persists at these denaturant concentrations. The disruption of the residual structure at higher GuHCl concentrations is shown by the loss of excimer formation with a transition midpoint at 3.4 M GuHCl (Fig. 5). As comparison the unfolding of py-N67C/C206-py monitored by far-UV CD at 222 nm shows a transition midpoint at 2.8 M GuHCl for unfolding of secondary structure (Fig. 3). We can conclude from our experiments that a compact structure is preserved between β -strands 3 and 7, which constitute the central part of the molecule. Our earlier experiments have shown that single amino acid residues in β -strands 3–5 are inaccessible to alkylation until the concentration of GuHCl is raised to about 5 M, which indicates the existence of a compact residual structure under these strongly denaturing conditions [4,5]. However, Cys-206 in β -strand 7 does become accessible for alkylation during the molten globule \rightarrow unfolded state transition, with a midpoint concentration of 2.5 M GuHCl. In the present investigation it was clear that this β -strand is also held together with the region consisting of β -strands 3–5. β -strand 7 most likely forms one of the borders of the stable residual structure. β -strands 3–5 are included in the large hydrophobic cluster in the major domain of HCAII, and β -strands 6–7 constitute the most hydrophobic stretches in the molecule [28]. In other proteins residual structure under strongly denaturing conditions has also been detected [6–10]. It has been pointed out that continuous strings of non-polar side chains, which are present in the proteins mentioned above as well as in HCAII, could nucleate the formation of hydrophobic clusters [10]. From immobilization of a spin-label during refolding of spin-labeled HCAII [29], we suggested in 1975 that hydrophobic β -strands might act as a folding initiation site in HCAII. Others have also hypothesized that stretches of apolar side chains can act as chain folding initiation sites [30]. Furthermore, in this study we detect a compact state that appears to remain at very high concentrations of GuHCl. The increase in monomer fluorescence indicates that the environment surrounding the pyrenes is further changed in the 4–7 M GuHCl range. The detected quenching would require a compact structure, irrespective of the nature of the quenching, at very high concentrations of GuHCl. Since no plateau

value is reached this compact unfolded state is preserved up to 7 M GuHCl.

The residual structure appears to have considerable flexibility, since according to an earlier study of Trp fluorescence [19] all Trp residues were exposed to solvent at 3 M GuHCl. In addition, a recent kinetic study [31] of refolding from 3.3 M GuHCl solutions indicated that Trp-97 situated in β -strand 4 becomes buried in a native-like environment within a 2 ms burst phase. Apparently, the residual structure can rearrange into a compact and rigid form very rapidly.

We can conclude that pyrene excimer fluorescence measurements provide structural information which complements that gained by chemical reactivity measurements and the use of single spectroscopic probes. The excimer approach is particularly suitable for probing compactness in extensively denatured proteins, i.e. for identifying residual structures. That is because the probes must be brought together in the 3-D structure to allow excimer formation from two fluorophores linked to the polypeptide chain. We will continue to map the boundaries of the residual structures by pyrene probing.

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