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Adsorption to silica nanoparticles of human carbonic anhydrase II and truncated forms induce a molten-globule-like structure

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Abstract Human carbonic anhydrase II pseudo-wild type ($HCAII_{pwt}$) and two truncated variants were adsorbed to ≈9 nm silica nanoparticles. Ellipsometry was used as an indirect measure of protein adsorption. The structural changes of adsorbed proteins were investigated with the use of circular dichroism (CD), intrinsic fluorescence, ANS binding ability and inhibitor binding capacity. It was found that the variants that were truncated at positions 5 and 17 in the N-terminal end attain a molten-globule-like state after interaction with the silica nanoparticles. In contrast, the more stable $HCAII_{pwt}$ retained most of its native structure after 24 h adsorption to silica nanoparticles. The result suggests that surface induced unfolding may give rise to intermediates similar to those for unfolding induced by, for example GuHCl. Thus, the intermediate observed has some features of the molten globule.

Key words: Human carbonic anhydrase II; Adsorption; Molten globule; Circular dichroism; Fluorescence; Nanoparticle

1. Introduction

Protein adsorption onto solid interfaces from aqueous solution is a well-known phenomenon that has been extensively investigated [1]. Proteins change their conformation when they adsorb from solution onto flat surfaces as monitored using different methods such as total internal reflectance fluorescence (TIRF) [2], circular dichroism (CD) [3,4], infrared (IR) spectroscopy [5] and antibody-binding ability [6].

Important questions that concern the adsorption process and structural alterations in the adsorbed proteins remain unanswered. For example, structural changes may be forced upon the protein by adaptation to the chemistry of the solid surface. On the other hand, structural changes may also occur due to intrinsic properties of the protein, roughly independent of the surface chemistry.

Norde has shown that some intrinsic properties determine the amount of structural change by dividing proteins into two classes, soft and hard [7]. He has shown that soft proteins undergo larger structural rearrangements upon adsorption than hard proteins. One way of altering intrinsic properties of proteins is to use site-directed mutagenesis. With this method it is possible to alter one amino acid at a time in the protein and to see how the alteration effects the adsorption behavior to a certain surface. This approach has been carried out for T4-lysozyme [8,9].

Human carbonic anhydrase II (HCAII) is an interesting model protein for this kind of studies due to the existence of several mutated versions of the enzyme and extensive folding studies of the protein [10–13,16].

HCAII is a monomeric zinc-enzyme, 29.3 kDa, which is dominated by a 10-stranded, open β-sheet (Fig. 1). The X-ray structure has been determined to a resolution of 1.56 Å [15]. Equilibrium unfolding studies of HCAII [16], demonstrated that there is a stable folding intermediate at moderate concentrations of guanidine hydrochloride (GuHCl), referred to as a 'molten globule' [17,18]. This equilibrium intermediate has a compact central core composed of a large hydrophobic cluster [16,19]. In addition, the seven tryptophans (Trp) residues in the HCAII protein structure have been extensively studied with fluorescence [20] and CD [21].

The variants used were HCAII pseudo-wild type ($HCAII_{pwt}$), Trunc 5 and Trunc 17. $HCAII_{pwt}$ has the naturally occurring cysteine in position 206 replaced by a serine. The spectroscopic properties and activity of this pseudo-wild type are apparently identical to those of the wild type. Trunc 5 and 17 have had their 4 and 16 N-terminal amino acids, respectively, removed using site-directed mutagenesis [11]. Deletion of the first 4 N-terminal residues causes unfolding of the helical segments comprising amino acid residues 13–18 and 21–24. The removal of an additional 12 residues does not introduce any additional unfolding. Furthermore, the truncation of 4 to 16 residues results in destabilisation by 4–5 kcal/mol relative to the intermediate state; the stability of the intermediate state relative to the unfolded state is not changed significantly [11].

The aim of this investigation was to characterize the conformation of HCAII adsorbed at silica nanoparticles. Different spectroscopic methods were used to register conformational changes of HCAII and the two truncated variants and those were compared to the properties of GuHCl-denatured HCAII.

2. Materials and methods

2.1. Chemicals

8-Anilino-1-naphthalenesulfonic acid (ANS) and 5-dimethylaminonaphthalene 1-sulfonamide (DNSA) were purchased from Sigma.

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; DNSA, 5-dimethylaminonaphthalene 1-sulfonamide; $HCAII_{pwt}$, pseudo-wild type of human carbonic anhydrase II with a C206S mutation; GuHCl, guanidine hydrochloride; Trunc 5, N-terminally truncated at position 5

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Guanidine hydrochloride (GuHCl) was obtained from Pierce and was of sequanal grade, its concentration being determined refractometrically [22]. The colloidal, negatively charged, silica particles (food grade quality) were kindly provided by EKA-Nobel, Stenungsund, Sweden. They were used without further modification apart from dilution in buffer. The stock solution contained 5.09*E*+17 particles/ml with an average diameter of 9 nm. All other chemicals were of reagent grade.

2.2. Protein mutation, production and purification

The mutants $HCAII_{pwt}$, Trunc 5 and 17 were produced using site-directed mutagenesis. Full details of mutagenesis, production and purification procedures are described in Aronsson et al. [11].

2.3. Adsorption procedure

All experiments were performed in 10 mM sodium/potassium phosphate buffer, pH 7.5 at 23°C. The protein samples were filtered (Millipore; pore size 0.45 μm) prior to use. The protein molecules were mixed with an equal amount of particles to obtain a 1:1 ratio of protein to particle. Spectroscopic measurements were made before addition of particles and then after 24 h. In some cases, 3.4 μM protein was unfolded with 1.5 M GuHCl for 24 h to induce a molten-globule state.

2.4. Ellipsometry

Ellipsometry is an optical method for the quantification of adsorbed thin organic layers at flat reflecting surfaces [23]. The ability of the proteins to adsorb at the nanoparticles was assessed with an indirect ellipsometry technique. Premixing protein and nanoparticles in a 1:1 ratio for 24 h resulted in the abolishment of any apparent adsorption to the flat silicon oxide surface. This was regarded as a safe indication that the protein was adsorbed to the nanoparticles and that the solution contained essentially no free protein.

2.5. CD measurements

CD spectra were recorded on a spectrodichrograph (Jobin-Yvoin Instruments SA, Longjumeau, France), employing constant N_2 flushing. The instrument was calibrated with an aqueous solution of d_{10}-(+)-camphorsulfonic acid at 290 nm. Each CD spectrum represents the average of three scans obtained by collecting data at 0.5 nm intervals with an integration time of 2 s. The three separate scans were inter compared before summation to detect possible alterations of the sample during the scan period. The protein spectra were corrected by a spectrum of a reference solution lacking the protein but otherwise identical. The ellipticity is reported as mean residue molar ellipticity ([θ], in degree cm^2 $dmol^{-1}$) according to Eq. 1:

$$[\theta] = [\theta]_{obs} \cdot mrw/10lc \quad (1)$$

where $[\theta]_{obs}$ is the ellipticity (degrees), mrw is the mean residue molecular weight (molecular weight 29 300 and 259 amino acid residues for $HCAII_{pwt}$), c is the protein concentration (g/ml) and l is the optical path length of the cell (cm).

Far-UV CD spectra were obtained by scanning the proteins in 10 mM potassium/sodium phosphate buffer, pH 7.5, in a 0.5 mm quartz cell. Near-UV CD spectra were recorded using the same buffer in a 5 mm quartz cell. The protein concentration was 17 μM in all experiments in the near- and far-UV regions.

2.6. Fluorescence measurements

Steady-state fluorescence emission spectra were recorded on a Hitachi F4500 spectrofluorophotometer. The final protein concentration was 3.4 μM in all experiments. The cuvette path length was 10 mm.

2.6.1. Intrinsic tryptophan fluorescence. The Trp residues were selectively excited at 295 nm. Excitation and emission bandwidths were 2.5 and 10 nm, respectively. The emission spectrum between 300 and 500 nm was recorded with and without nanoparticles as indicated.

2.6.2. ANS binding. A 200-fold molar excess of ANS (680 μM) was used over the protein concentration. The samples were excited at 360 nm and the spectrum between 450 and 650 nm was registered. Excitation and emission bandwidths were 2.5 and 10 nm, respectively.

2.6.3. DNSA binding. A 10-fold molar excess of DNSA (34 μM) was used over the protein concentration. The sample was excited at 320 nm and the spectrum was recorded between 400 and 600 nm. Excitation and emission bandwidths were 5 and 10 nm, respectively.

3. Results and discussion

It has been suggested that the primary contributions to the overall driving force for adsorption come from, structural rearrangements in the protein molecule, dehydration of the protein and sorbent surfaces, and redistribution of charged groups in the interfacial layer [1]. Thus protein adsorption is strongly associated with structural changes of the protein. It has also been proposed that the stability of the protein structure is important for the adsorption-desorption behavior on different surfaces [24]. Proteins are reported to stick harder to a surface if the three-dimensional structure has a low stability [8]. This is understandable if changes in the protein structure are more allowed for in a less stable protein. A way to investigate this is to use a structurally well characterized protein and to monitor the structural rearrangements upon adsorption. Site-directed mutagenesis can be used to construct variants that are less stable than the wild-type protein. $HCAII_{pwt}$ is known to form a stable equilibrium folding intermediate of the molten-globule type [16] at moderate concentrations of GuHCl and it also forms compact residual structures at high concentrations of GuHCl. We have earlier characterized these partially denatured states in detail using a combination of protein engineering, chemical modification and spectroscopic techniques [10,13,16,19,20]. The question in this study is if the GuHCl-denatured states of $HCAII_{pwt}$ and the less stable variants Trunc 5 and 17 have structural properties in common with their adsorbed states.

3.1. Probing hydrophobic patches by ANS binding

The hydrophobic dye, ANS, is widely used to identify the molten globule state [17,25]. This state is compact and has solvent-exposed hydrophobic surfaces to which ANS can bind, and as a consequence thereof, the emission spectrum increases in intensity and becomes blue-shifted [26]. The ANS spectra of free and adsorbed $HCAII_{pwt}$ are shown in Fig. 2a, and the wavelength maxima are given in Table 1. The intensity increased by 40% and the spectrum was blue-shifted 6 nm after equilibration for 24 h with particles. For

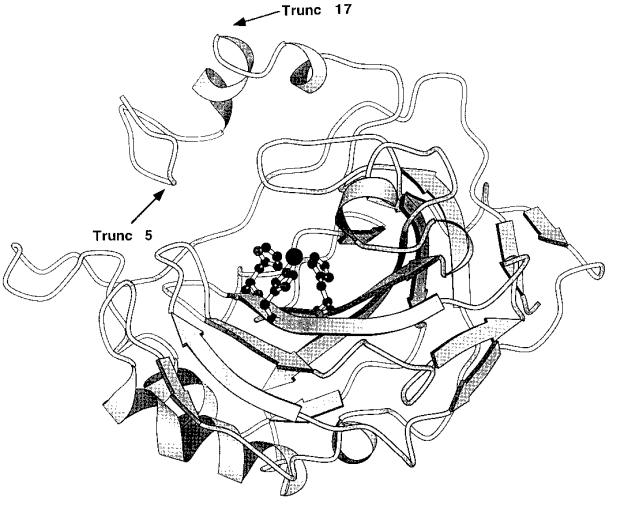

Fig. 1. Schematic drawing of the polypeptide backbone of HCAII with the site of the truncations marked by arrows. The figure was produced using the program Molscript [14] and the coordinates were kindly provided by Dr. Kjell Håkansson.

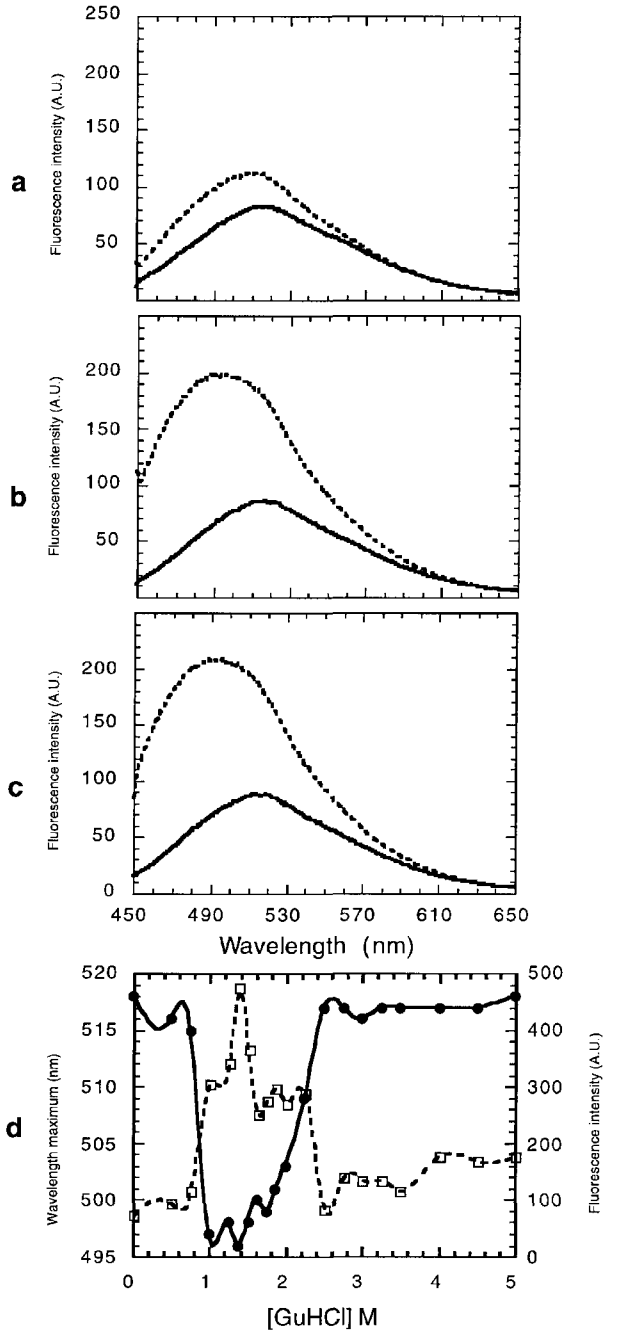

Fig. 2. Binding and subsequent fluorescence of the hydrophobic probe ANS to $HCAII_{pwt}$ and to mutants thereof. (a) $HCAII_{pwt}$. (b) Trunc 5. (c) Trunc 17. Protein without particles (———) and after 24 h equilibration with particles (- - -). (d) GuHCl-induced unfolding of $HCAII_{pwt}$ in 0.1 M Tris-HCl buffer, pH 7.5, as monitored by ANS fluorescence. The wavelength of maximum intensity (●—●) and the intensity at this wavelength (□- - -□) are both represented in this graph.

Trunc 5 and 17 the effects were more profound (Fig. 2b,c); a 22 nm blue-shift was observed in the ANS fluorescence and the increase in fluorescence intensity was 140 and 135%, respectively. Fig. 2d shows GuHCl-induced denaturation of $HCAII_{pwt}$ as monitored by ANS. From a maximal blue-shift of 22 nm and an approx. 4-fold increase in fluorescence intensity, it is evident that ANS binds to the molten-globule intermediate that dominates in the 1–2 M GuHCl region.

From the magnitude of the blue-shift it seems as if adsorbed Trunc 5 and Trunc 17 adopt a compact structure with exposed hydrophobic patches, which appear very similar to the $HCAII_{pwt}$ molten globule. However, the fluorescence intensity of the adsorbed Truncs did not increase to the level of the molten globule. It is possible that some hydrophobic patches were attached to the surface of the particle and therefore not accessible for ANS. The effects on the ANS spectrum upon adsorption of $HCAII_{pwt}$ were small and a comparison with the GuHCl-denaturation curve indicates that the adsorption was accompanied by a local unfolding process that led to exposure of a minor hydrophobic surface. (Alternatively, a small fraction of $HCAII_{pwt}$ might have been denatured to the molten-globule state upon adsorption.)

3.2. CD studies

3.2.1. Near-UV CD spectrum. To investigate how the tertiary structure of the protein was affected by the particles, the CD spectrum in the near-UV region was studied. The near-UV spectrum of $HCAII_{pwt}$ is mainly composed of bands that arise from the seven Trp residues when they become fixed in an asymmetric environment in the native structure [21]. This spectrum is lost at 1.3 M GuHCl [16], indicating that the specific tertiary structure has vanished in the molten-globule intermediate. When $HCAII_{pwt}$ was exposed to nanoparticles, the characteristic CD bands remained, but the intensity of the whole spectrum was uniformly decreased after 24 h (Fig. 3a). This probably indicates that a minor fraction of the protein molecules has undergone a conformational change. In contrast, the spectra for the truncated variants, after 24 h exposure to the nanoparticles, show that the asymmetry around the aromatic residues was lost (Fig. 3b,c). Since the Trp residues are distributed to various parts of the protein structure, the result indicates that most or all of the tertiary structure was ruptured, again indicating a structure which is molten-globule like.

3.2.2. Far-UV CD spectrum. To investigate how the secondary structure in the protein was influenced by the adsorption to the particles, the spectrum in the far-UV region was recorded with and without particles. As has been reported previously [21], the spectrum in the far-UV region does not only reflect the secondary structure in HCAII, since Trp residues also contribute in this wavelength region. This is important to know when evaluating the results from these measure-

Table 1
Fluorescence emission maxima

	Intrinsic tryptophan fluorescence			ANS fluorescence			DNSA fluorescence		
	$HCAII_{pwt}$	Trunc 5	Trunc 17	$HCAII_{pwt}$	Trunc 5	Trunc 17	$HCAII_{pwt}$	Trunc 5	Trunc 17
Without particles	334	336	333	516	515	516	461	470	471
With particles[a]	338	343	342	510	493	492	461	522	521

Values are expressed in nm.
[a]After 24 h equilibration with nanoparticles.

ments. The far-UV CD spectra for $HCAII_{pwt}$, Trunc 5 and Trunc 17 are shown in Fig. 4a,b and c, respectively. The changes observed in the spectrum for $HCAII_{pwt}$ were relatively small and may indicate an altered environment around one or two Trp residues or alternatively reflect a minor loss of secondary structure. The spectra of the truncated variants change dramatically upon adsorption onto the nanoparticles. However, comparison with reference spectra [27] indicates a substantial contribution from secondary structure elements to these spectra, which further supports the notion that the structural properties of the adsorbed Truncs are very similar to those of the GuHCl-denatured intermediate.

3.3. Probing the active site by DNSA binding

By measuring changes in the fluorescence of an extrinsic active-site probe, DNSA, it is possible to observe structural changes in the active site. The binding of the DNSA probe is critically dependent on the specific structure in the active site. Hence, when the native active site is altered, DNSA looses its affinity. When DNSA dissociates from the active site the emission spectrum becomes red shifted and the intensity drops [28]. For $HCAII_{pwt}$ only a small drop in the fluorescence intensity and a minor change in the wavelength of maximum

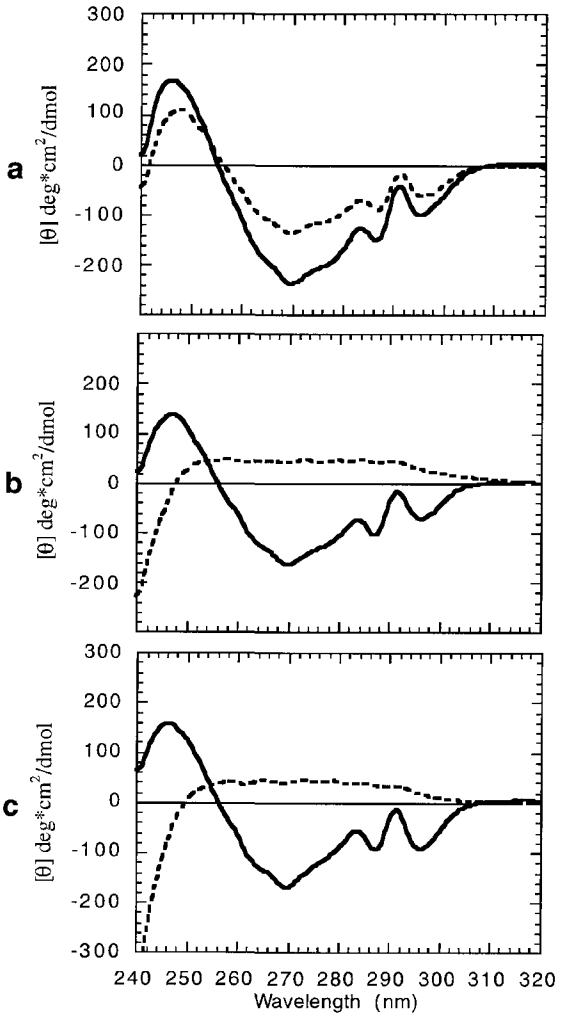

Fig. 3. Near-UV CD spectra of HCAII variants. Protein without particles (———) and after 24 h equilibration with particles (- - -). (a) $HCAII_{pwt}$. (b) Trunc 5. (c) Trunc 17.

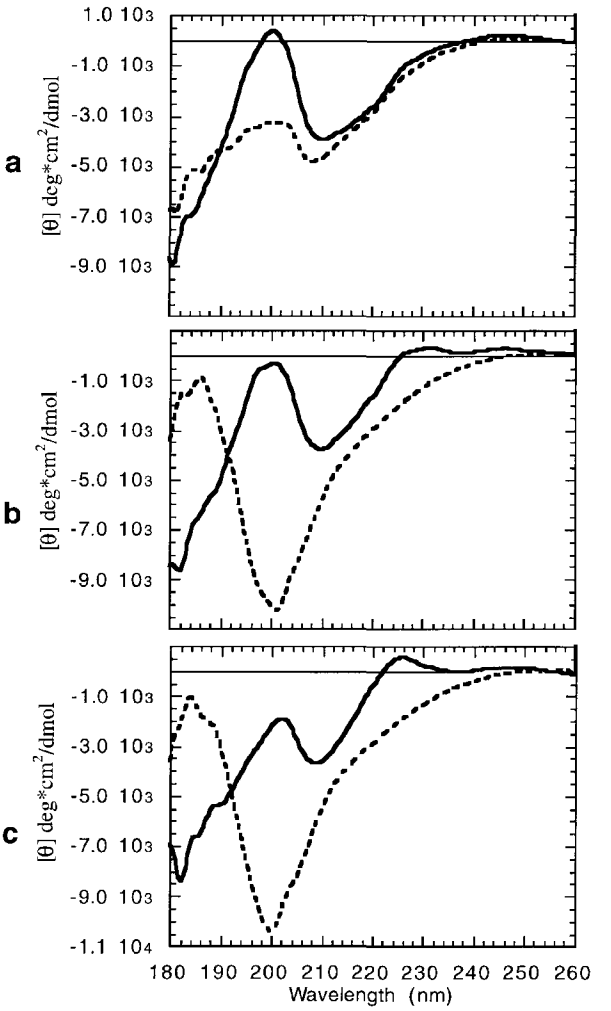

Fig. 4. Far-UV CD spectra of HCAII variants. Protein without particles (———) and after 24 h equilibration with particles (- - -). (a) $HCAII_{pwt}$. (b) Trunc 5. (c) Trunc 17.

intensity were observed (Fig. 5a). This suggests that the structural alterations observed in Fig. 2a, Figs. 3a and 4a are small enough to leave the active site intact.

For the truncated variants of HCAII a large decrease in fluorescence intensity (Fig. 5b,c) and a considerable red shift of the wavelength maximum were observed (Table 1). This indicates rupture of the native conformation of the active site. It has previously been reported that the activity disappears and that DNSA binding is abolished in the GuHCl-induced transition to the molten-globule intermediate [29].

3.4. Intrinsic tryptophan fluorescence

If $HCAII_{pwt}$ is excited at 295 nm the intrinsic fluorescence from the seven Trp residues is selected. The individual contributions from these Trp residues have been determined recently [20], showing that Trp 97 and 245 are the two major emitters, and that there is a complex energy transfer between the Trp residues with internal quenching in the protein structure.

The steady-state fluorescence emission spectra of $HCAII_{pwt}$ and the truncated forms were measured with and without particles. The wavelength of maximum intensity for the intrinsic tryptophan fluorescence of $HCAII_{pwt}$ is 352 nm for the

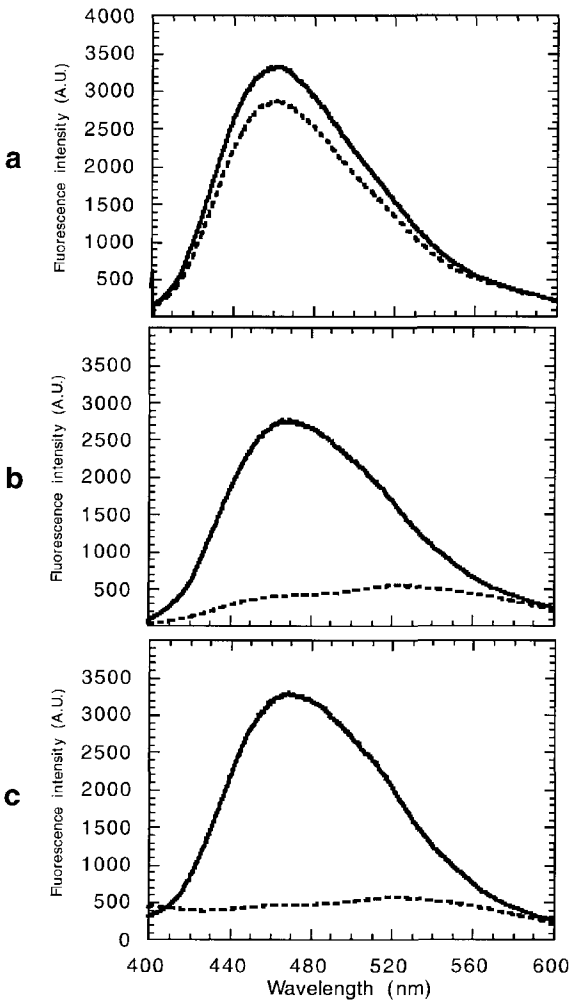

Fig. 5. Structural changes in the active site of HCAII as observed by monitoring the fluorescence of an extrinsic active-site probe, DNSA. The protein without particles (——) and after 24 h equilibration with particles (- - -). (a) $HCAII_{pwt}$. (b) Trunc 5. (c) Trunc 17.

denatured and 343 nm for the molten-globule state [20]. This emission maximum for the adsorbed state of $HCAII_{pwt}$ (Table 1) is intermediately located between the maxima for the native and the molten-globule state. Interestingly, the emission maxima for Trunc 5 and 17 in 1.5 M GuHCl (342 and 341 nm, respectively) are very similar to those of the adsorbed state (Table 1).

4. Conclusions

The conformational changes of $HCAII_{pwt}$ are probably small upon adsorption to the nanoparticles, as probed by the conformational parameters used in this study.

The main reason to the observed different conformational effects caused by the adsorption process between $HCAII_{pwt}$ and the Truncs is most likely due to differences in stability of the structure of the variants. From our data it is not likely that the structural differences of the native states caused by the truncations give rise to the adsorption-mediated conformational changes. The rationale is that adsorption causes very similar structural effects when only 4 amino acid residues are removed as when a large part (16 residues) of the N-terminal domain is truncated. Truncation of 4 or 16 amino acid residues leads to very similar degree of destabilization, and thus appears to be the factor responsible for how extensive the unfolding will be upon adsorption.

As judged from our measurements the conformations between the adsorbed Truncs and the GuHCl-induced folding intermediate of $HCAII_{pwt}$ are very similar, allowing us to make structural comparisons. The GuHCl-induced intermediate state has previously been structurally characterized in some detail [10,16,19,20]. Thus, the interior hydrophobic core of the adsorbed Truncs should be compact and a large part of the predominant β-structure (β-strands 3–7), which is located in this region, should retain their native-like structure. In contrast, a more flexible structure is expected in the peripheral β-strands, indicating that the stability of the secondary structure is less in the outer parts than in the center of the protein. Furthermore, the active-site region is disrupted, leading to an enzymatically inactive molecule.

It is clear that structural consequences upon adsorption to nanoparticles can be further clarified by a systematic use of well-characterized mutants with different stabilities.

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