Transient Interaction with Nanoparticles "Freezes" a Protein in an Ensemble of Metastable Near-Native Conformations†

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ABSTRACT: It is well-known that adsorption of proteins on interfaces often induces substantial alterations of the protein structure. However, very little is known about whether these conformational changes have any consequence for the protein conformation after desorption from the interface. To investigate this matter, we have selected a protein−particle system in which the enzyme human carbonic anhydrase I (HCAI) alternates between the adsorbed and free state upon interaction with the silica nanoparticles. High-resolution NMR analysis of the protein with the particles present in the sample shows a spectrum that indicates a molten globular-like structure. Removal of particles results in refolding of virtually all HCAI molecules to a fully active form. However, the two-dimensional NMR analysis shows that refolding does not result in a single well-defined protein structure but rather provides an ensemble of protein molecules with near-native conformations. A detailed comparative chemical shift analysis of 108 amide signals in $^{1}H-^{15}N$ HSQC spectra of native and desorbed HCAI reveals that the most profound effects are located at β-strands in the center of the molecule. The observation of very slow H−D exchange in the central β-strands of HCAI [Kjellsson, A., Sethson, I., and Jonsson, B. H. (2003) *Biochemistry 42*, 363−374] in conjunction with our results indicates that the kinetic barriers for conformational rearrangements in the central core of the protein are low in the presence of nanoparticles but are very high under native conditions.

Interactions of proteins with and adsorption of proteins to solid surfaces are of interest in diverse applications, such as protein purification, the food industry, and medicine (*1*). Many of the mechanisms involved in protein adsorption from a macroscopic perspective have been thoroughly described, and the behavior of different proteins has been shown to be related to their specific surface properties, i.e., the distribution of different amino acid residues. In particular, it has been shown that the protein's stability affects both the rate and extent of conformational alteration upon adsorption to a solid surface (*2*−*7*).

Recently, several groups have published results from high-resolution solution NMR[1] investigations of small peptides bound to a solid-state material in solution (*8*, *9*) and protein−particle systems that have provided information about protein−surface interactions at the level of individual residues (*4*, *10*, *11*). In addition, hydrogen−deuterium experiments have been used to obtain information about conformational changes (*12*−*15*). Several proteins have been shown to adopt a molten globular-like state when they interact with solid surfaces (*2*−*4*, *12*). An interesting issue raised by the major observed conformational changes is whether the desorbed proteins regain their native conformation or if the interaction introduces persistent structural alterations. To investigate this matter, we have selected a protein−particle system in which the protein alternates between adsorbed and free states. In an earlier study (*4*), it was established that the interaction between human carbonic anhydrase I (HCAI) and silica nanoparticles fulfills this requirement. The complete backbone assignment of HCAI (*16*) allows us to use NMR to monitor small alterations in the proteins' native structure at the level of individual residues.

In this study, we report results of NMR analyses of HCAI both during incubation with nanoparticles, and after removal of the particles from the solution by centrifugation. We have shown in recent studies (*4*, *10*) that the protein's interaction with particles induces a conformational shift toward a molten globular-like state. In this study, we show that upon removal of the particles HCAI refolds to a fully active state. However, the NMR spectra show that the refolding results in an ensemble (at least three different states) of protein molecules with near-native conformations. The observed heterogeneities are mainly located in the central core of the enzyme and persist for at least 1 week. High-resolution studies of this type therefore give interesting information concerning the energy landscape of proteins that interact with surfaces. The observations also indicate that interactions with various media used for protein purification may have prolonged structural effects on the protein's conformation.

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[1] Abbreviations: HCAI, human carbonic anhydrase I; NMR, nuclear magnetic resonance spectroscopy; HSQC, heteronuclear single-quantum correlation.

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MATERIALS AND METHODS

Silica Particles. The colloidal, negatively charged, silica particles (food grade quality) used in this study were kindly provided by EKA-Chemicals (Stenungsund, Sweden). Before being used, the particles were extensively dialyzed against sample buffer [20 mM TRIS and 20 mM NaCl (pH 8.4)]. The particles are stable in solutions at pH >8 and low to moderate salt concentrations (*17*). The stock solution contained 5.09×10^{17} particles/mL with an average diameter of 9 nm.

Samples. All protein samples in this work were held at 30 °C when incubated with the nanoparticles. To hinder bacterial growth, all samples contained 0.02% azide. The protein concentrations were determined from the absorbance of samples at 280 nm using an extinction coefficient of 46 800 M^{-1} cm^{-1}. Analysis on an SDS gel of the protein before and after incubation with particles shows virtually identical results with only one intact protein band at ~30 kDa.

NMR Experiments. Uniformly ^{15}N-labeled HCAI was produced and purified as described in ref *4*. The NMR samples consisted of 0.3 mM protein in 20 mM TRIS buffer with 20 mM NaCl at pH 8.4 and with a 90:10 H_2O:D_2O ratio. The temperature was held at 30 °C. For samples with particles, the stoichiometric ratio of protein to particle was 1:1. The two-dimensional (2D) NMR experiments were performed with a Varian Inova Unity 600 spectrometer equipped with a triple-resonance (^1H, ^{13}C, and ^{15}N) cryoprobe with pulsed field gradients (PFG) along the *z*-axis using 2D HSQC in the sensitivity-enhanced gradient version, as described in ref *18*. The acquisition time was 205 ms with 4K data points and 256 complex t_1 increments. The 2D data were processed using nmrPipe (*19*), and the spectra were analyzed using SPARKY (*20*).

Centrifugation. To remove the particles, the samples were centrifuged at 20 °C for 4.5 h at 15 000 rpm (23 646 rcf), and the supernatants were transferred to new sample tubes and centrifuged for an additional 4.5 h at 15 000 rpm (23 646 rcf). It has earlier been shown that centrifugation at 5161 rcf for 20 h efficiently sediments all particles (*4*).

Enzyme Activity Measurements. Enzyme activity was measured by following the hydrolysis kinetics of the substrate *p*-nitrophenyl acetate (pNPA) at a wavelength of 405 nm using a Varian Cary 100 Bio UV−visible spectrophotometer, as described by Thorslund et al. (*21*).

RESULTS AND DISCUSSION

Particles Induce Side Chain Flexibility and Conformational Changes. This study concerns the properties of the protein HCAI after desorption from a solid silica surface achieved by complete removal of silica nanoparticles by centrifugation. This initial section gives a background about the experimental conditions and the current knowledge of the protein−nanoparticle system. Earlier near-UV CD studies (*4*) have shown that more than 90% of the HCAI molecules adopted a molten globular-like tertiary structure after incubation for 6 days with silica nanoparticles. Notably, the CD measurements were taken for both bound and unbound protein molecules because the small size of the nanoparticles allows the use of absorption spectroscopy (*22*−*24*); i.e., the nanoparticles (9 nm diameter) do not scatter light in the UV region. In this study, a NMR measurement has been performed on samples under conditions identical to those for the CD measurements having a 1:1 ratio of silica nanoparticles to protein. The spectrum for the intact protein−nanoparticle sample after incubation for 6 days shows no native or near-native peaks (see Figure 1b) and is similar to the spectrum of the GuHCl-induced molten globule (*25*). Notably, the signals from all six N-bound indole protons (from the tryptophan side chains) merge to a common shift (indicated with an arrow in Figure 1b), suggesting that the side chains of the tryptophans interact with the surrounding media; i.e., the tertiary structure around the side chains is altered, allowing high mobility. The NMR spectrum most probably emanates from protein molecules that are free in solution because when bound to the nanoparticle (425 kDa) the protein−nanoparticle complex tumbles very slowly, leading to a broadening of the resonances beyond detection in a HSQC experiment. Taken together, the NMR and CD results show that both bound and unbound proteins have adopted molten globular or near-molten globular structures after 6 days. Earlier studies of the equilibrium between the bound and unbound protein by gel permeation and analytical ultracentrifugation have shown that the protein exchanges

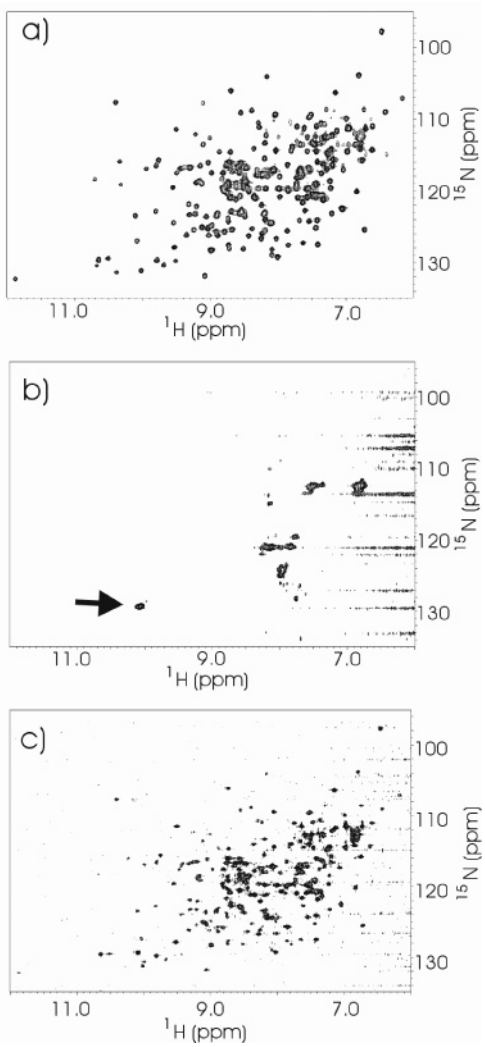

FIGURE 1: NMR spectra of HCAI that has not been treated with particles (a) and HCAI after incubation for 6 days with 9 nm particles before (b) and after (c) removal of the particles from the solution. The arrow indicates the combined Trp−indole NH peak.

between the bound and free states (*4*). Apparently, after incubation for 6 days, the protein molecules cannot refold to a nativelike structure during the time they are free in solution when the nanoparticles are present in the solution.

Lingering Effects on Soluble Proteins. An interesting issue raised by the observation that prolonged incubation of HCAI with silica particles induces major structural alterations toward a molten globule-like state (*4*) is whether the desorbed protein can refold to its native state or becomes trapped permanently in a non-native conformation. To investigate this matter, HCAI was incubated with particles for various times, after which the particles and particle-bound protein were removed from the soluble protein fraction by centrifugation. The samples containing soluble protein were subsequently investigated by enzyme activity assays and 2D NMR. Since we have earlier shown in CD experiments (*4*) that the length of time that the protein is incubated with particles affects the degree of structural alteration, two samples were selected for characterization by NMR: one with a short incubation time (10 min) and the second with a long incubation time (6 days) with the particles.

The NMR spectrum obtained from the sample incubated for a short time before removal of the particles is indistinguishable from a spectrum of the native protein, as illustrated by the partial, superimposed spectra in Figure 2 (top panel). Thus, it is apparent that a short incubation with the particles does not result in persistent structural changes in the soluble protein.

The NMR spectrum of the sample retrieved after the long incubation with particles appears to be very similar to a native spectrum, as shown in panels a and c of Figure 1. However, there are numerous small, but significant, shift differences as can be seen in the partial spectra in the panel B and panel C of Figure 2. From analysis of 108 well-resolved backbone resonances, the effects on the spectrum can be divided into the following types.

(a) Many (*32*) of the resonances remain at their original position (shifted fewer than 6 standard deviations) in the spectrum for the native protein, as can be seen in Figure 2 (panel B and panel C). Another 27 resonances have shifted between 6 and 12 standard deviations from their original positions.

(b) Some (*5*) resonances shift too far from their original positions to be assigned (or are broadened beyond detection), as illustrated by the resonance at arrow 1 in Figure 2 (panel B and panel C). Broadening beyond detection would imply a mobility higher than that in the native structure. Missing resonances because of large chemical shifts do not imply that they have shifted outside the observation window but instead imply that they are shifted into regions of the spectrum that are very crowded with overlapping resonances, and therefore, they are not observed.

(c) Some resonances (*44*) show a comparatively large and significant shift (more than 12 standard deviations from their original positions), as illustrated by the resonance indicated by arrow 2 in Figure 2 (panel B and panel C).

(d) For some resonances (*7*), a fraction of the population remains at the "native" position and another fraction is shifted significantly, as indicated by arrow 3 in Figure 2 (panel B and panel C). This is the most obvious indication that the spectrum emanates from an ensemble of near-native structures. Further indications that several different structures exist

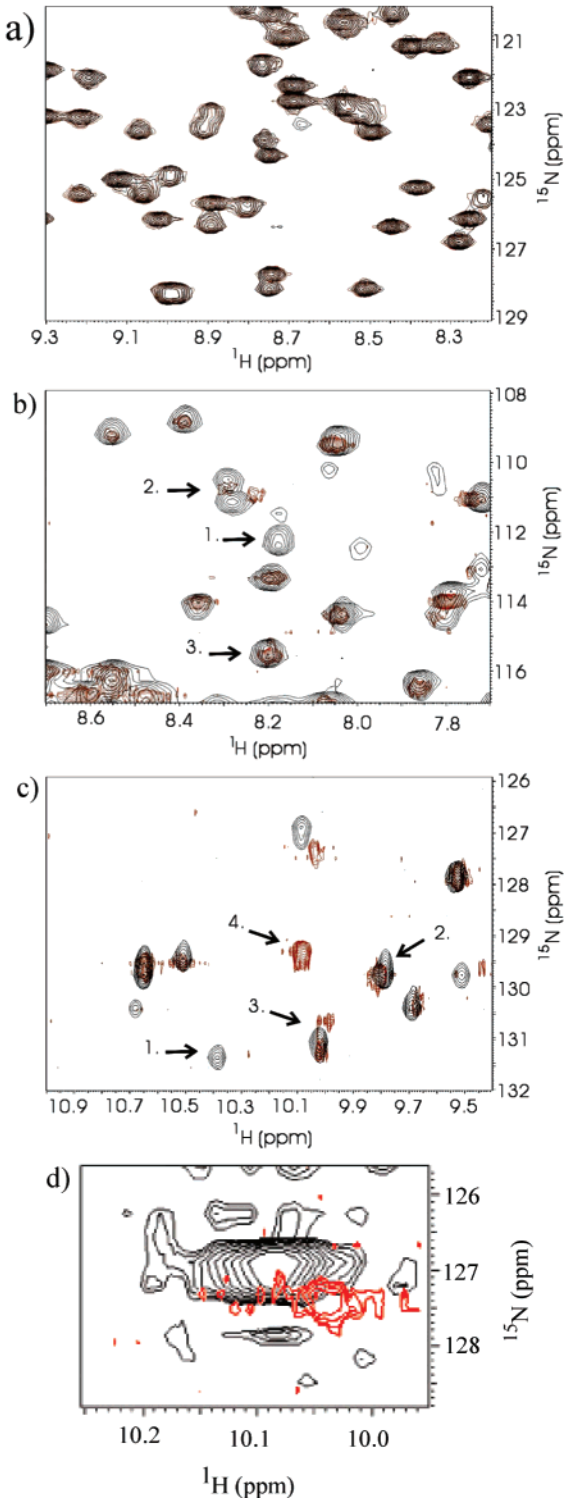

FIGURE 2: Partial NMR spectra from samples that have not been treated with particles (black peaks) superimposed (red peaks) with spectra from samples that have been incubated with particles for 10 min (a) or 6 days (b and c). The data show that no conformational changes occur in the protein after incubation for 10 min (a). After 6 days, some signals are unaffected but others are shifted markedly. Arrow 1 denotes a signal that has been completely shifted, arrow 2 a signal that has been partly shifted, and arrow 3 a signal in which part of the signal has been shifted, but the main part is still at the native shift, indicating that some (but not all) of the molecules have adopted a different conformation at this residue. Arrow 4 denotes the combined indole proton peak. (d) Comparison at one typical resonance plotted at the noise level showing that the untreated sample (black resonance) does not contain any contribution from molecules with near-native structure (red resonance).

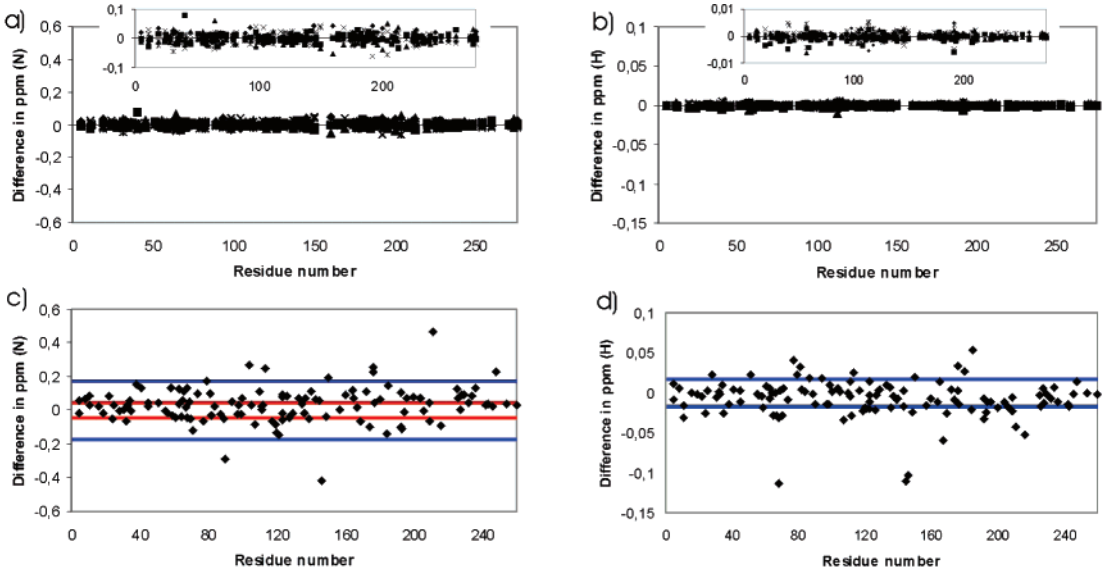

FIGURE 3: Shift differences for HCAI incubated at 30 °C for various times with and without particles. Panels a and b show shift variations for three samples of HCAI without particles measured at five different time points, with a blowup inserted to illustrate the five different shift value for each monitored residue. Panels c and d show shift differences (compared to an untreated sample) for HCAI in a sample that was incubated for 6 days at 30 °C with particles and which was subsequently centrifuged. In panel c, lines representing 6 standard deviations (red) and 12 standard deviations (blue) are present, and in panel d, lines representing 12 standard deviations (blue) are present.

come from the observation that some signals (*6*) split into three or more parts.

(e) A fraction of the Trp−indole NH resonance appears at the typical position for the indole−NH resonance in bulk water, as shown by arrow 4 in Figure 2 (panel C). This fraction corresponds to an ~40% lowered intensity for each of the indol resonances, Trp 123 and 209; i.e., 60% of the signals remain in their native positions, showing heterogeneity around these Trp residues in the post-treatment sample. Interestingly, the associated amide shifts for these residues show only small deviations from their native positions. The intensities of the resonances for the other Trp indole NH group are unaffected within the error of the intensity measurements.

The NMR measurements on the centrifuged sample were repeated each day for 7 days after removal of nanoparticles. A comparison showed that the NMR spectra did not change in this time. Thus, the disturbances of the protein structure as revealed by NMR persist for at least 7 days.

Are the Observed Shift Differences Significant? To investigate if the reported shift differences were significant, or merely results of normal statistical variation or small, unknown experimental differences, five control experiments were performed. For this purpose, three samples containing HCAI were prepared at three different times (with no incubation with particles) and their spectra were recorded. For two of these samples, spectra were recorded again several weeks later, as shown in panels a and b of Figure 3. It should be pointed out that the observed shift differences are not due to small pH differences between the samples, which has been checked. A comparative analysis of these spectra shows that the shift variation was very small indeed: less than approximately ±0.005 ppm in the ^{1}H dimension and approximately ±0.05 ppm in the ^{15}N dimension. A similar analysis of the shift differences between an untreated HCAI sample and a sample of HCAI that had been incubated with silica nanoparticles for 6 days shows much larger shift differences. Here, the shift deviation is up to approximately ±0.1 ppm in the proton dimension and up to approximately ±0.4 ppm in the ^{15}N dimension, as shown in panels c and d of Figure 3, respectively.

One might argue that the observed large shift differences are not the result of interaction with the nanoparticles but originate from a purification procedure leading to a sample with nonhomologous structures which would contain a mixture of molecules with native or near-native protein structures, the argument being that upon interaction the particles should preferentially bind to the native protein molecules leaving only near-native protein molecules free in solution. If that were the case, the NMR resonances of the original sample would contain the spectrum from this fraction also. That is so because the original protein sample is prepared and incubated under conditions concerning all experimental parameters identical to those of the sample with nanoparticles. Since the NMR measurements of the samples are performed with identical parameters, a comparison of the spectra at a level where the noise is visible would reveal if the original sample contained any substantial fraction of near-native proteins. Comparisons of the spectra (illustrated in Figure 2d) show no trace of the near-native molecules in the original sample. Note that no enrichment, only selection, can occur in this experimental system because it does not contain any step in which the absolute concentration of a particular protein fraction can be increased by a concentrating procedure. Thus, an increase in a fraction having a particular conformation can only occur by a transformation from another conformational state; i.e., direct interaction with the particles is necessary for production of near-native conformations.

Particle-Induced Conformational Changes Compared with H−D Exchange Analysis. A conservative assumption is that shift differences of fewer than 6 standard deviations can be considered as native shifts and that shift differences larger than 12 standard deviations unambiguously can be considered as non-native shifts. When the backbone signals that shifted more than 12 standard deviations in either the N or the H

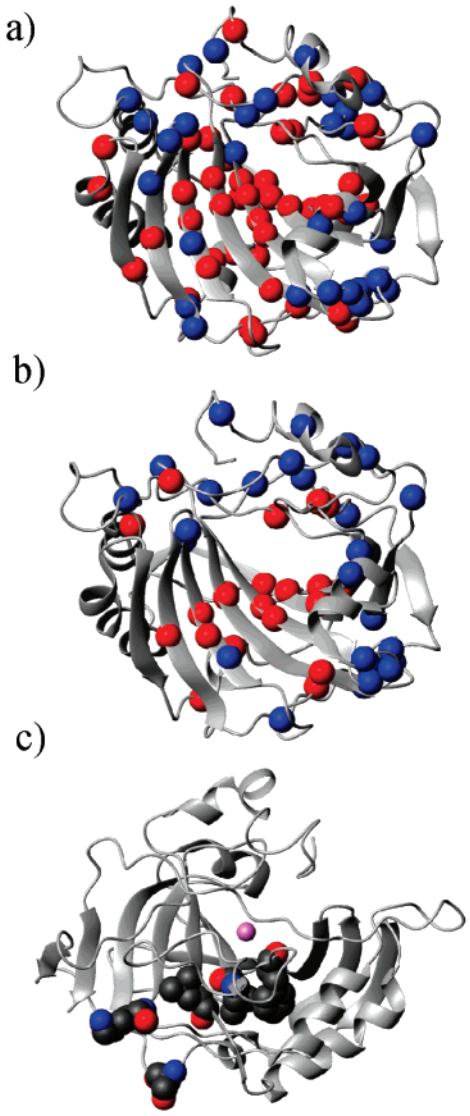

FIGURE 4: (a): Three-dimensional structure of HCAI, showing the location of amides for which the signals shifted less than 5 Hz in both H and N dimensions (blue spheres, 32 residues) and more than 10 Hz in either or both of these dimensions (red spheres, 53 residues). (b) Three-dimensional structure showing amide protons for which data are available from both particle-incubated HCAI experiments (this work) and H−D exchange experiments on HCAI (*25*). Amides represented by blue spheres exchange their protons immediately and are shifted less than 5 Hz; those represented by red spheres do not exchange their protons during the experiment time and are shifted more than 10 Hz. (c) Three-dimensional structure of HCAI, showing the residues that have shifted too far or broadened too much for detection. Also, colored purple is the active site Zn^{2+} ion. The representation is rotated 180° compared with panels a and b. This figure was prepared using MOLMOL (*32*).

dimension or both following incubation with the particles (red spheres in Figure 4) and the signals that shifted fewer than 6 standard deviations in both dimensions (blue in Figure 4) are highlighted in the three-dimensional structure, an interesting pattern is revealed. It is apparent that almost all observed signals from the central β-sheet (β-strands 5−8 from left to right in Figure 4) shifted more than 12 standard deviations; i.e., amino acids in this region can be trapped in metastable non-native conformations. In contrast, the shifts from the N-terminal minidomain, the loops, and many surface-exposed amino acids are predominately native; i.e., those parts appear to rapidly adopt a native structure after removal of the particles. Four of the five residues with resonances that are shifted or broadened beyond detection (highlighted in Figure 4c) are directly involved with the large hydrophobic cluster that is associated with the central β-strands. This observation further strengthens the argument that the central portion of the protein has high energy barriers toward rearrangements when the main parts of the protein have adopted nativelike conformations. Interestingly, the intensity of the resonances from the two Trp side chains that extend into the central area is strongly affected, while the Trp side chains closer to the surface are virtually unaffected. Thus, the observations on the side chains corroborate the conclusion from the observation on the backbone NH resonances.

To analyze if these observations are correlated to the stability and dynamics in the different regions of HCAI, we compared these findings with the results from H−D exchange experiments on the same protein (*25*). In the report from Kjellson et al. (*25*), it was shown that the majority of the amide groups in the peripheral parts of the protein readily exchange their protons, while the amide protons in the central β-strands were very resistant to exchange when the protein was maintained under native conditions. Thus, their results clearly show that the peripheral parts are quite dynamic, and that the center of the β-sheet is highly stable, considerably reducing the probability of large conformational changes; i.e., the very slow exchange rates for the protons in the central β-strands indicate that the backbone and side chains in these β-strands are in a rigid environment.

Several different observations indicate that the structure of HCAI is substantially altered upon prolonged incubation with silica nanoparticles. The observation of a severely perturbed NMR spectrum from HCAI in the presence of particles (Figure 1b) indicates that the interaction strongly perturbs both the tertiary and secondary structure. Measurements of both near- and far-UV spectra of HCAI in the presence of particles have also detected large effects on the tertiary structure and a perturbation of the secondary structure (*4*). These findings show not only that the tertiary structure is strongly perturbed but also that the central β-strands are affected by the interaction with particles. Hence, when a HCAI molecule dissociates from a particle, it will have a disturbed structure throughout the protein. While the N-terminus, loops, and peripheral secondary structures seem to adopt a nativelike conformation relatively quickly after desorption from the silica particles, large proportions of the amino acids from the central β-strands seem to be trapped in an ensemble of partially non-native conformations. These observations together agree well with the H−D exchange data, which show that the loops and the N-terminus are highly dynamic under native conditions, while the central β-sheets are much less dynamic (*25*). Panel B in Figure 4 shows those residues for which the data from both H−D exchange and particle treatment correlate, i.e., amides with slow H−D-exchange and large shifts (red spheres) are found in the central β-strands, and amides with rapid H−D exchange and native shifts (blue spheres) are found closer to the protein surface. Hence, residues in loops and close to the protein surface with high dynamics seem to rapidly adopt a native conformation after interaction with the particle. The striking observation is that the residues in the central

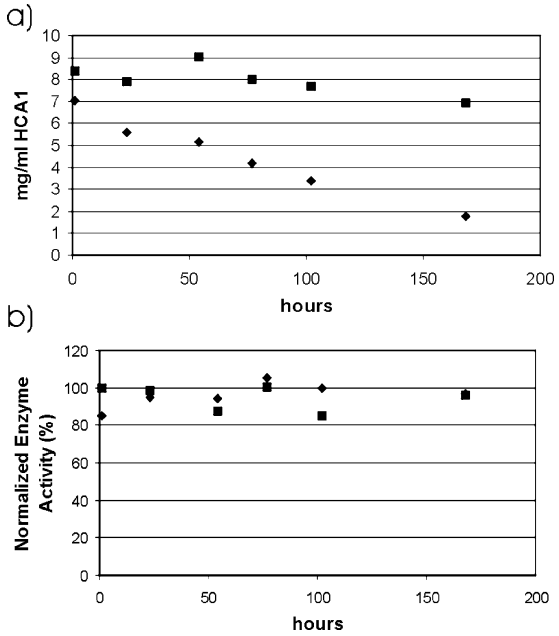

FIGURE 5: (a) Proportion of HCAI that does not sediment with the particles during centrifugation after different incubation times (◆). Squares show data for reference samples that were not incubated with particles. (b) Normalized enzyme activity (using the activity of the native enzyme as 100%) for the samples after the particles have been removed by centrifugation (using the same symbols as in panel a).

β-strands that have a non-native conformation at the time of dissociation from the particles will not readily adopt a native conformation, due to the weak dynamics in this part of the protein; i.e., the kinetic barriers for rearrangement in this central core of HCAI are very high in a nativelike environment.

The Perturbed HCAI Molecules Are Fully Catalytically Active! Activity assays of the protein/particle solution after different incubation times show that catalytic activity decreases with the length of incubation, indicating that proteins are not active during interaction with the particles (data not shown). To investigate if the observed structural perturbations in the free protein molecules affect the catalytic efficiency, samples were withdrawn from the particles after different periods of incubation and their enzymatic activity was measured. As can be seen in Figure 5a, increasing proportions of the HCAI molecules sediment with the particles upon centrifugation as the incubation time increases. However, as can be seen in Figure 5b, the specific enzyme activity of the protein remaining in solution after centrifugation is identical (within the error limit of the measurements that is less than 10%) to that of the native protein; i.e., this shows that more than 90% of the protein molecules that have adopted metastable near-native conformations are fully active. The location of the active site is indicated in Figure 4c by the catalytic Zn^{2+} ion that is ligated to histidine residues that are found in central β-strands 4 and 5. Interestingly, numerous studies on active site mutants of human carbonic anhydrases indicate that full catalytic activity requires a very precise positioning of catalytically important residues (*26*−*30*). Apparently, an intact positioning of catalytic residues in the active site is compatible with differing conformations in the nearby core region of the enzyme. It is interesting to note that Vamvaca et al. (*31*) recently showed that a molten globular ensemble of the enzyme chorismate mutase has considerable enzymatic activity.

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