Tertiary Structure Formation at Specific Tryptophan Side Chains in the Refolding of Human Carbonic Anhydrase II[†]

Per Jonasson,[‡,||] Göran Aronsson,[‡] Uno Carlsson,[§] and Bengt-Harald Jonsson*[,‡]

Department of Biochemistry, Umeå University, S-901 87 Umeå, Sweden, and IFM-Department of Chemistry, Linköping University, S-581 83 Linköping, Sweden

Received July 29, 1996; Revised Manuscript Received February 18, 1997[⊗]

ABSTRACT: The refolding reaction of human carbonic anhydrase II has been characterized by use of seven variants in which tryptophan residues have been replaced by Phe or Cys, in each case giving proteins with six tryptophans. Intrinsic tryptophan fluorescence was used to monitor the refolding in the 2 ms−60 s time range, and kinetic traces showing the contributions from each particular tryptophan were obtained by calculation of differences between the wild-type protein and the variants. Earlier assignment [Mårtensson, L.-G., Jonasson, P., Freskgård, P.-O., Svensson, M., Carlsson, U., & Jonsson, B.-H. (1995) *Biochemistry 34*, 1011−1021] of specific fluorescence properties to each tryptophan, especially regarding energy transfer and intrinsic fluorescence quenching, has made it possible to use the kinetic data to describe the formation of tertiary structure at defined tryptophan residues. In summary, it was found that tertiary structure is formed earlier at those tryptophans that are associated with the central core of β-strands than at tryptophan residues in the N-terminal minidomain.

Refolding of human carbonic anhydrase II ($HCAII_{pwt}$),[1] from the guanidine hydrochloride unfolded state, reveals a complex pattern of kinetic phases when monitored by intrinsic tryptophan fluorescence. There are both increases and decreases in fluorescence intensity, and the changes occur on time scales from milliseconds to several minutes. Since the protein contains seven tryptophan residues, the spectral overlap has made it impossible to determine which tryptophans contribute to these various changes in fluorescence intensity.

One way to get more specific information from intrinsic fluorescence refolding studies is to replace a tryptophan with a less fluorescent amino acid residue, in the case of $HCAII_{pwt}$ leaving six tryptophans. The difference in the tryptophan fluorescence between such a mutant and the wild-type protein would report on the fluorescence of the removed tryptophan. However, the analysis must take into account the possibilities of energy transfer between tryptophan side chains and of quenching of fluorescence by neighboring amino acid side chains. In a recent study, we succeeded in assigning specific fluorescence properties to each particular tryptophan residue in $HCAII_{pwt}$ (Mårtensson et al., 1995). It is especially noteworthy that the fluorescence for three of these (Trp5, Trp123, and Trp209) is essentially fully quenched by nearby amino acid side chains when the protein has adopted its native structure, but the quenching is absent in a partly unfolded state and in an extensively unfolded form of the protein. Thus, the quenching of the fluorescence of these tryptophan side chains can be used as a sensitive indicator of native-like tertiary structure.

In the present study, we have taken advantage of the assignment of well-defined fluorescence properties to each tryptophan. The kinetic fluorescence trace of $HCAII_{pwt}$ has been compared to the traces of the seven different tryptophan mutants. As a result, we obtained kinetic traces corresponding to each particular tryptophan residue. The analysis includes a description of the formation of tertiary structure at each tryptophan location, giving information on intermediate structures in the folding process.

Human carbonic anhydrase II is a structurally and functionally well-defined protein; it is a zinc containing enzyme which catalyzes the interconversion of carbon dioxide and water into bicarbonate and protons (Liljas et al., 1994). It is a monomeric, single-domain protein with a molecular mass of 29 300, and its X-ray structure has been resolved to 1.54 Å (Håkansson et al., 1992). HCAII has some helical structure and a dominating β-sheet which extends throughout the entire molecule (Figure 1).

MATERIALS AND METHODS

Chemicals. Guanidine hydrochloride (GuHCl, Sequanal grade) was purchased from Pierce, and its concentration was determined refractometrically (Nozaki, 1972). Tris(hydroxymethyl)aminomethane (reagent grade) was obtained from Sigma.

Protein Production and Purification. The reference in this study, $HCAII_{pwt}$, is a variant in which the wild-type cysteine in position 206 is replaced by serine (Aronsson et al., 1995). All mutants lack this cysteine as well. Site-directed mutagenesis, protein production, and purification were carried out as previously described (Mårtensson et al., 1995).

[†] This work was supported by grants from the Swedish National Board for Industrial and Technical Development (U.C. and B.-H.J.), the Swedish Natural Science Research Council (U.C. and B.-H.J.), "Magn. Bergwalls Stiftelse" (B.-H.J.), "Sven and Lilly Lawskis Fond" (G.A.), and "J C Kempes Minnes Stipendiefond" (P.J. and G.A.).

* Address correspondence to: Bengt-Harald Jonsson, Department of Biochemistry, Umeå University, S-901 87 Umeå, Sweden. Phone: +46-90165283. Fax: +46-90167661. E-mail: nalle@chem.umu.se. URL: http://www.biochem.umu.se/.

[‡] Umeå University.

[||] Present address: Department of Chemistry and Process Technology, Mid-Sweden University, S-851 70 Sundsvall, Sweden.

[§] Linköping University.

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: $HCAII_{pwt}$, pseudo wild-type human carbonic anhydrase II; GuHCl, guanidine hydrochloride; CD, circular dichroism.

S0006-2960(96)01882-X CCC: $14.00

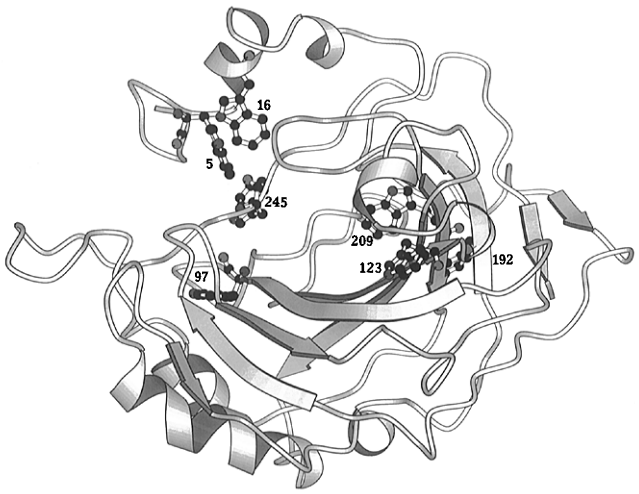

FIGURE 1: Schematic representation of the polypeptide backbone of human carbonic anhydrase II. The seven tryptophans are displayed as balls and sticks. The figure was produced using the program Molscript (Kraulis, 1991).

Kinetic Measurements. The enzymes (55 μM) were denatured by incubating in 3.3 M GuHCl, 0.1 M Tris−H_2SO_4, pH 7.5, at 23 °C for 1 h. Renaturation was achieved by diluting the denatured enzymes 11-fold into buffer, giving a GuHCl concentration of 0.3 M and a protein concentration of 5 μM. Extra Zn^{2+} was not added because it has been found that the reactivation kinetics and yield are unaffected by excess Zn^{2+} (Ikai et al., 1978; Bergenhem & Carlsson, 1989). The changes in intrinsic fluorescence were monitored with an Applied Photophysics SX-17MV stopped-flow instrument equipped with 2.5 mL and 250 μL syringes to allow 10:1 mixing. With this configuration, the experimental dead time was 2 ms, as determined by using the reaction between 2,6-dichlorophenolindophenol and L-ascorbate (Tonomura et al., 1978). The excitation wavelength was 295 nm with a bandwidth of 5 nm, and the emission intensity was recorded at 340 nm with a bandwidth of 7 nm. The cuvette was thermostated to 23 °C. Fast kinetics (<1 s) were recorded with an instrumental time constant of 2 ms and slower kinetics (>1 s) with a 150 ms time constant. The SX-17MV does not allow measurements for much longer than a minute, due to diffusion between the mixer and the cuvette.

In a different experiment, 17.5 μM of $HCAII_{pwt}$ was incubated in 4.5 M GuHCl, 0.1 M Tris-H_2SO_4, pH 7.5, at 23 °C for 1 h. Refolding was initiated by a 3.5-fold dilution to 5 μM $HCAII_{pwt}$ and 1.3 M GuHCl.

Data Analysis. All data were fitted with nonlinear least-squares analysis, using the computer program GraFit (Erithacus Software Ltd., Staines, U.K.). The fits are the least number of added exponentials needed to give randomly distributed residuals.

Structure Analysis. Analysis of the protein structure was done on a computer graphics system using the program Insight II (Biosym Technologies, San Diego, CA). The coordinates, from X-ray crystallography data, were kindly provided by Håkansson et al. (1992).

RESULTS

$HCAII_{pwt}$ unfolds in two global transitions, as monitored by fluorescence, when subjected to increasing concentrations of GuHCl (Mårtensson et al., 1995), and the last transition is virtually complete at 3 M GuHCl. In refolding experiments, when diluting from the GuHCl-unfolded state (3.3 M) to native conditions (0.3 M), there is first a large increase in fluorescence within the 2 ms dead time of the experiment (Table 1). The amplitude of this burst phase is 90% of the difference in intensity between the native and denatured state. After the dead time, one can distinguish five exponential phases, three of which were monitored in the stopped-flow apparatus (Figures 2 and 3). At first there is a small, but significant, increase in intrinsic fluorescence intensity with a rate of 12 s^{-1} ($t_{1/2}$ = 58 ms). This increase is followed by two larger exponential decreases with rates of 0.34 s^{-1} ($t_{1/2}$ = 2 s) and 0.040 s^{-1} ($t_{1/2}$ = 17 s). On a yet slower time scale there is a biphasic increase in fluorescence (phases 4 and 5). These phases are best studied in experiments using manual mixing of reagents, and have been described earlier (Aronsson et al., 1995); they have rates of 0.15 min^{-1} ($t_{1/2}$ = 4 min) and 0.043 min^{-1} ($t_{1/2}$ = 17 min), and their amplitudes (normalized to the amplitudes in the present study) are 0.05 and 0.16, respectively.

In another experiment, $HCAII_{pwt}$ was diluted from 4.5 to 1.3 M GuHCl, a concentration at which an equilibrium intermediate folding species is highly populated (Mårtensson et al., 1995). In this experiment (Figure 2), the burst phase has decreased in amplitude, which led to a larger amplitude of the first phase than in the original experiment (Table 1). This increase in fluorescence is followed by a decrease which is best described by a single exponential. This last phase is slow and ends up on a fluorescence level of 0.95, a level at which, in the original experiment, only the first phase is completed.

With the tryptophan mutants, however, only experiments where the GuHCl concentration was diluted from 3.3 M to the native conditions of 0.3 M were carried out. To obtain the kinetic trace emanating from a particular tryptophan, we calculated the difference between the kinetic trace of $HCAII_{pwt}$ and the trace of the mutant lacking that particular tryptophan. Previously, we calculated such differences at 5 M GuHCl and equilibrium (Mårtensson et al., 1995), and we found that each tryptophan contributed roughly equally to the intensity of $HCAII_{pwt}$ (Trp209 contributes slightly more than the mean value and Trp245 slightly less). In the present study, we got similar results for the start conditions at 3.3 M GuHCl (Table 1); i.e., adding the contributions of each individual tryptophan produces the intensity of $HCAII_{pwt}$. The reason to select 3.3 M GuHCl as the initial unfolding condition was twofold. First, when the denaturation is monitored by fluorescence from tryptophans, we observe two transitions of which the second is complete at 3 M GuHCl (Mårtensson et al., 1995). Second, earlier results, from measurement of solvent accessibility for engineered cysteine residues, have shown that unfolded $HCAII_{pwt}$ contain a residual compact structure at 3−4 M GuHCl, which is gradually denatured in a broad transition between 4 and 8 M GuHCl (Mårtensson et al., 1993; Svensson et al., 1995). Thus by unfolding at 3.3 M GuHCl, we select a reasonably well-defined initial state that is not within any unfolding transition that is presently known.

Trp97 and Trp209 contribute more than the other five tryptophans to the burst phase (Table 1). These two tryptophans account for one-third each of the amplitude in $HCAII_{pwt}$. Again, the amplitudes of all the individual tryptophans add up to the burst-phase amplitude of $HCAII_{pwt}$.

Table 1: Kinetic Parameters from Fluorescence Refolding Experiments of $HCAII_{pwt}$ (Here Denoted pwt) and the Differences between $HCAII_{pwt}$ and Each of the Seven Tryptophan Mutants[a]

	F_{denat} (±0.02)	Amp_{burst} (±0.04)	k_1 (s^{-1}) (±2)	Amp_1 (±0.002)	k_2 (s^{-1}) (±0.04)	Amp_2 (±0.002)	k_3 (s^{-1}) (±0.007)	Amp_3 (±0.002)
pwt[b]	0.58	0.38	12	0.02	0.34	−0.06	0.040	−0.08
W5F[b]	0.48	0.34	6	0.03	0.22	−0.07	0.024	−0.10
W16F[b]	0.48	0.37	5	0.03	0.24	−0.08	0.028	−0.08
W97C[b]	0.49	0.26	15	0.01	0.25	−0.06	0.048	−0.06
W123C[b]	0.48	0.34	8	0.01	ND	ND	0.050	−0.03
W192F[b]	0.50	0.35	9	0.02	0.15	−0.04	0.021	−0.07
W209F[b]	0.47	0.26	4	0.01	ND	ND	ND	ND
W245C[b]	0.54	0.34	ND	ND	ND	ND	0.018	−0.05
pwt-W5F[b]	0.10	0.04	ND	ND	ND	ND	ND	ND
pwt-W16F[b]	0.10	0.01	ND	ND	ND	ND	ND	ND
pwt-W97C[b]	0.09	0.12	ND	ND	ND	ND	ND	ND
pwt-W123C[b]	0.10	0.04	ND	ND	0.30	−0.06	0.037	−0.05
pwt-W192F[b]	0.10	0.03	ND	ND	0.41	−0.05	ND	ND
pwt-W209F[b]	0.11	0.12	ND	ND	0.36	−0.06	0.067	−0.07
pwt-W245C[b]	0.04	0.04	ND	ND	0.31	−0.07	0.071	−0.05
pwt (inter.)[c]	0.58	0.32	5	0.10	0.018	−0.045	ND	ND

[a] Standard deviations are given in parentheses. [b] Dilution from 3.3 to 0.3 M GuHCl. [c] Dilution from 4.5 M to 1.3 M GuHCl.

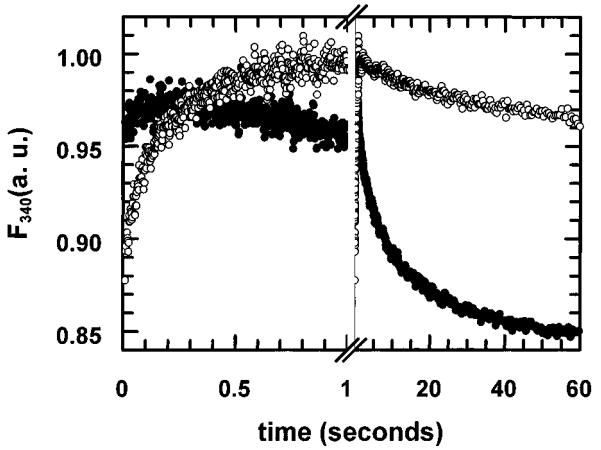

FIGURE 2: Changes in intrinsic tryptophan fluorescence of $HCAII_{pwt}$ upon refolding from unfolded to native conditions (●). 55 μM $HCAII_{pwt}$ was incubated in 3.3 M GuHCl, 0.1 M Tris−H_2SO_4, pH 7.5, at 23 °C for 1 h. Renaturation was achieved by rapidly diluting 11-fold to 0.3 M GuHCl and 5 μM protein. The other trace (○) shows the changes in intrinsic tryptophan fluorescence of $HCAII_{pwt}$ upon refolding from denaturing conditions to a GuHCl concentration promoting an intermediate state. 17.5 μM $HCAII_{pwt}$ was incubated in 4.5 M GuHCl, 0.1 M Tris−H_2SO_4, pH 7.5, at 23 °C for 1 h. Renaturation was achieved by a rapid 3.5-fold dilution to 1.3 M GuHCl and 5 μM protein.

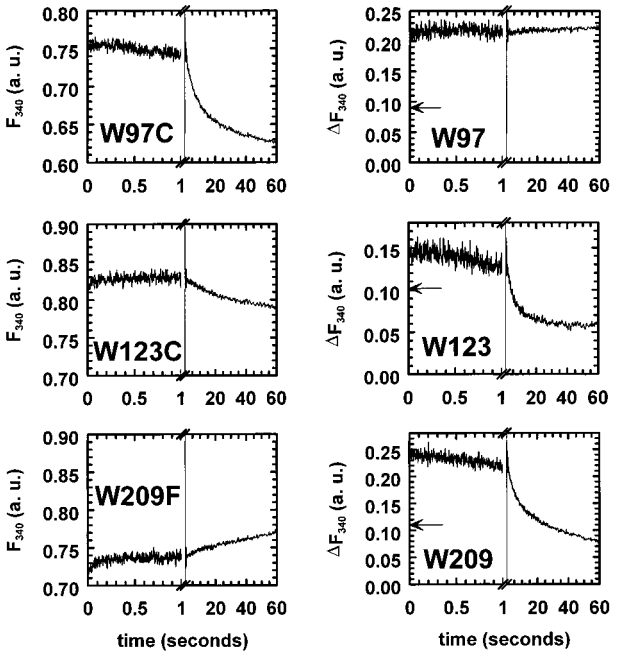

FIGURE 3: The observed kinetic traces for $HCAII_{pwt}$ and the tryptophan mutants W97C, W123C, and W209F (left column). Individual kinetic traces of tryptophans W97, W123, and W209 (right column), which were obtained by subtracting the kinetic trace of each tryptophan mutant from the trace of $HCAII_{pwt}$.

It may be noted that the amplitudes are calculated from differences between kinetic traces. The observation that the sum total of the amplitudes equals the burst amplitude of $HCAII_{pwt}$ is a strong indication that experimental conditions were very well-defined in all measurements and that the differentiation method is robust. It also indicates that $HCAII_{pwt}$ and all the mutants have similar structural properties upon completion of the burst phase.

The experiments on $HCAII_{pwt}$ and the mutants were always performed back-to-back using identical conditions, including the instrumental settings. Moreover, the experiments, which were repeated several times, gave virtually identical results regarding both amplitudes and rates, and, thus, it is reasonable to believe that the calculated kinetic traces describe the events at each particular tryptophan. A concern might be that the approach of obtaining individual kinetic traces by calculation of differences between the wild-type and mutant traces would amplify nonsystematic errors. However, an alternative approach would be to use the raw data directly, and compare the phase amplitudes of the wild-type with those of the mutants. As can be seen in Table 1, both approaches give the same picture of which tryptophans contribute to the phases. In fact, by comparing differences in amplitudes for the k_2 and k_3, we find those to be similar to the corresponding amplitudes obtained by fitting the difference of the kinetic traces.

In the first observable phase, after the instrumental dead time, the signal-to-noise ratio is too low to determine which tryptophans, if any, contribute more than others to the small fluorescence intensity increase one sees in $HCAII_{pwt}$. After summation of all the difference traces, one can note that a second after initiation of folding the individual contributions still add up to the intensity of $HCAII_{pwt}$; i.e., energy transfer between tryptophans and quenching of tryptophan fluores-

cence is not prominent at this stage.

Some tryptophans, namely, Trp5, Trp16, and Trp97, do not change their fluorescence to any significant degree during the time interval 1−60 s (Table 1, Figure 3). Trp123, Trp209, and Trp245 decrease their fluorescence biphasically with rates in the range 0.3−0.4 s^{-1} ($t_{1/2} \sim 2$ s) for one phase and 0.04−0.07 s^{-1} ($t_{1/2} \sim 13$ s) for the other (Table 1). The amplitudes of both phases for these three tryptophans are roughly equal in size. Trp192 exhibits only one phase in this time range with a rate of 0.4 s^{-1}. From one second and onwards, the individual tryptophan traces do not add up to the reference, $HCAII_{pwt}$. As we will argue, the tryptophans at this stage no longer fluoresce as separate entities but are involved in energy transfers and are affected by specific quenchers. The quenching of fluorescence by neighboring amino acid residues has made it possible to probe formation of native-like tertiary structure at Trp123 and Trp209.

DISCUSSION

Structural Perturbations Caused by Mutagenesis. When replacing an amino acid by site-directed mutagenesis, there is always a concern that the mutation will cause long range effects on the structure, especially when one replaces a large amino acid such as tryptophan; in the present study, the seven tryptophans in $HCAII_{pwt}$ were replaced by other amino acids. 2D NMR spectra of these ^{15}N-labeled tryptophan mutants have shown that there are no major structural disturbances in the surroundings of the six remaining tryptophan residues from the positions where the mutations are made in the various tryptophan mutants (Mårtensson et al., 1995; P. Jonasson, unpublished results). Additional evidence for this came from a circular dichroism study (Freskgård et al., 1994) where the spectrum of each tryptophan mutant was subtracted from the spectrum of $HCAII_{pwt}$. When these seven CD difference spectra were added to produce a composite spectrum, it exhibited all features of $HCAII_{pwt}$ in the near-UV.

In the analysis of the kinetic data, we have calculated the differences between the kinetic traces of $HCAII_{pwt}$ and the various mutants and we have used these differences to describe the folding at each individual tryptophan residue (Figure 3, Table 1). Alternatively, a direct comparison of phase amplitudes in the original kinetic traces of the mutants with the corresponding amplitudes of $HCAII_{pwt}$ can be used, because our analysis shows that it gives the same information. For these approaches to be valid, each of the mutants and $HCAII_{pwt}$ must fold as the same protein, i.e., their global kinetics must be identical. Stopped-flow far-UV CD measurements would have been ideal for that purpose, but, unfortunately, the far-UV CD spectrum of $HCAII_{pwt}$ is of low intensity and the interpretation in terms of secondary structure is severely complicated by large contributions from the tryptophan residues (Freskgård et al., 1994). Instead, we have chosen to use the raw data from the present study as an indicator of global folding (Figure 3, Table 1). The rationale is that each mutant protein contains 6 tryptophan residues and if the folding is unperturbed by the mutation the kinetic phases of $HCAII_{pwt}$ should be observed for the mutants, albeit with different amplitudes. As can be seen in Figures 2 and 3 and in Table 1, all mutants except W209F show folding kinetics that are very similar to those of $HCAII_{pwt}$. For the mutant W209F, the kinetic phases 2 and 3, that were observed for the other mutants, are missing andthe observed slow increase in fluorescence reflects the contributions of Trp5 and Trp16 that dominate the last two phases (Aronsson et al., 1995). Apparently, the energy transfer and quenching properties in this mutant are such that the contributions from the other tryptophan residues of the major domain vanish in phase 2 and 3. Therefore, these data do not show whether or not the W209F mutant folds as the wild-type protein. However, the reactivation, which occurs in the same time domain as the slowest kinetic phases measured by fluorescence (Aronsson et al., 1995), is virtually identical for $HCAII_{pwt}$ and the W209F mutant (data not shown). Similar results have been obtained for the mutants at Trp16, 97, and 245 (Dick Andersson, personal communication). Hence the intermediate state that is formed after phases 2 and 3 should be similar for $HCAII_{pwt}$ and all these mutants. Although arguments, that consider the initial burst phase and the very last slow phases, indicate that the W209F mutant folds similarly to $HCAII_{pwt}$, all interpretations concerning the W209F mutant should at this stage be considered as tentative.

Formation of Tertiary Structure at Various Tryptophan Side Chains. In a recent study (Mårtensson et al., 1995), we assigned specific fluorescence properties of all the individual tryptophans in the native protein and also of the proteins subjected to various concentrations of denaturant. We will use these assignments to aid in the interpretation of the kinetic data.

The Burst Phase: Trp97 and Trp209. Refolding of the protein is initiated by dilution of the GuHCl-denatured protein to conditions that favor formation of native structure. Initially, the fluorescence increases rapidly in a large burst phase that is completed within 2 ms, which is the dead time of the experiment. The rapid increase of the fluorescence in the burst phase most probably indicates a hydrophobic collapse of the protein leading to complete or partial burial of several tryptophan residues in an apolar environment. In particular, approximately one-third of the burst phase originates from Trp97, which in the native state is participating in a large cluster of thirty-four apolar residues, eight of which are aromatic (Figure 4B). The results show (Table 1, Figure 3) that Trp97 finds a native-like environment already within the 2 ms of the burst. That is because the fluorescence from Trp97 remains constant during all subsequent folding steps. However, since Trp97 is completely buried by apolar residues in the native state it may suffice that Trp97 becomes buried in an apolar cluster to restore its native-like fluorescence properties, and therefore, the Trp97 side chain is not necessarily locked in a well-defined conformation at this stage but might remain rather mobile. The observation that the fluorescence intensity remains constant after the burst phase might also indicate that the cluster is essentially dehydrated when the burst phase is over, at least in the region around Trp97. Hence, the core, in which Trp97 is located, must be relatively dense and, despite the lack of information regarding its spectral shift, we might speculate that water is not allowed to penetrate, which is thought to occur at least in the peripheral parts of a molten globule (Ptitsyn, 1992). As illustrated in Figure 4B, it seems as if a minimum requirement for formation of a dense and apolar environment of Trp97 would be that the apolar residues on β-strands 2−6 are clustered. Since Trp97 is situated in the periphery of such a cluster, it is probably

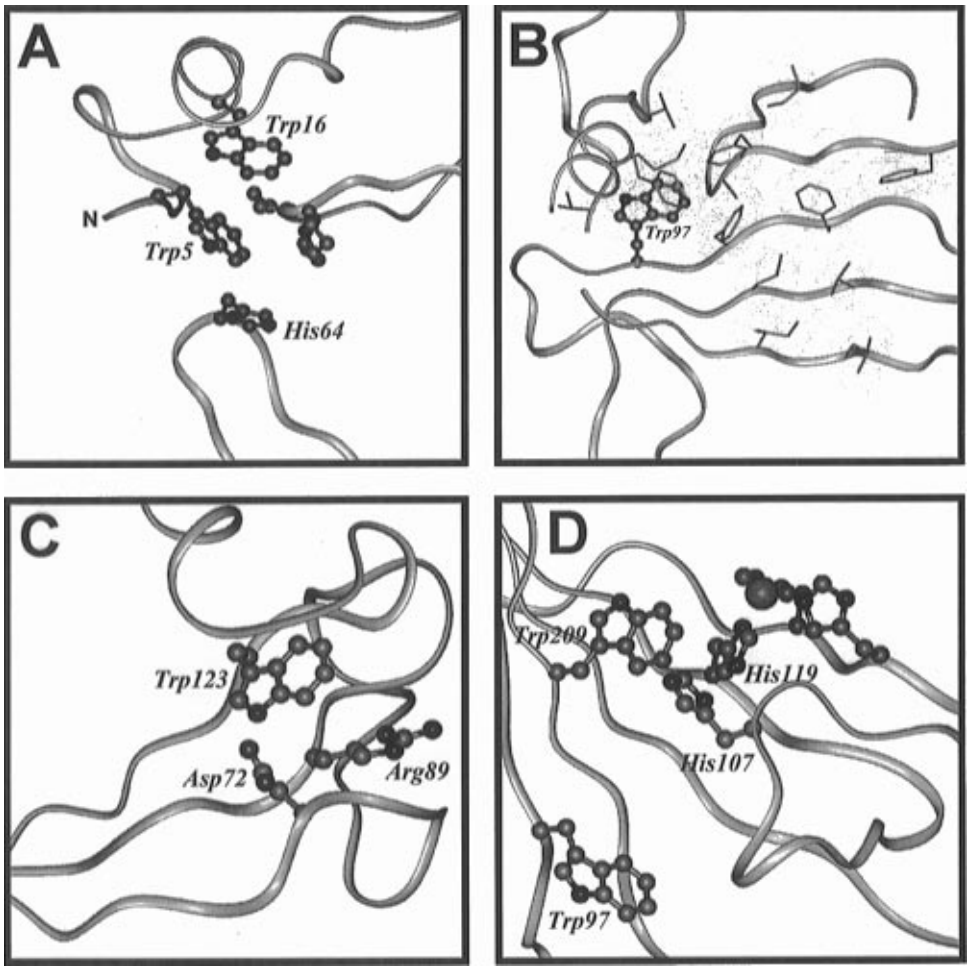

FIGURE 4: Schematic drawings of some features in the surroundings of tryptophans in $HCAII_{pwt}$. His64, situated in a loop between β-strands 2 and 3, quenches the fluorescence of Trp5 in the native state (A). A turn comprising Thr199, Pro200, and Pro202 is also essential for the native conformation of the N-terminus. Trp97 is situated in a large hydrophobic cluster, and a major part of the cluster is made of side chains on the β-sheet-containing amino acid segment 56−150 and the α-helix-containing segment 220−242 (B). The native fluorescence of Trp123 is quenched by Arg89 and possibly by Asp72 (C). Trp209 is situated on β-strand 7 (D), and putative quenchers are His107 (in a loop between β-strands 4 and 5) and His119 (on β-strand 5 coordinating the active-site zinc ion together with His94 and His 96). Trp92, situated on β-strand 8 (D), probably transfers its fluorescence to Trp209 in the native state.

needed that the helix-containing segment 220−242 with residues Phe226, Leu229, Met241, and Val242 also participates to create the proper apolar environment.

Trp209 contributes another one-third of the amplitude of the burst phase, and the remaining one-third comes from small contributions from the remaining five tryptophans. Since the fluorescence from each of these six tryptophan residues changes, in one or more of the kinetic phases that succeed the burst, none of them finds a native-like environment within the time of the burst phase.

When observing the intrinsic tryptophan fluorescence upon refolding of $HCAII_{pwt}$, we can distinguish five separate kinetic phases after the initial burst phase. The two slowest of these phases (phases 4 and 5) have been thoroughly described before (Aronsson et al., 1995). This study showed that formation of a native structure at the N-terminus constitutes the last steps in the folding, and it was also noted that the changes (a large increase) in fluorescence in these phases could be attributed solely to Trp5 and Trp16.

Trp245. Interestingly, the results of the present study modify the earlier interpretation regarding the role of Trp245 (Aronsson et al., 1995). The former study clearly showed that Trp245 found its final fluorescence properties within 60 s of refolding in those cases when it was situated in a truncated variant lacking Trp5 and Trp16 and in a full-length variant where Trp5 and Trp16 were replaced by phenylalanines. However, since the fluorescence from Trp245 is not quenched by nearby residues, it is not possible from those results to determine if its fluorescence is modified in the slow phases. When comparing the amplitude of Trp245 at 60 s with that observed by Mårtensson et al. (1995) of Trp245 in the native protein, it is clear that Trp245 contributes to the increase of the fluorescence in the slow phases. In the native $HCAII_{pwt}$ protein, Trp245 is in close proximity to Trp16 (Figure 1). Apparently, the docking of the N-terminal domain onto the central domain alters the structure at Trp245 when the residue at position 16 is tryptophan but not if it is phenylalanine.

Trp5 and Trp16. Trp5 and Trp16 are neighbors in the native protein, and their fluorescence properties are tightly coupled. In the native state, Trp5 is specifically quenched by His64 (Mårtesson et al., 1995) which resides in a short (four amino acids) loop between β-strands 2 and 3 (Figure 4A). The side chains of the Trp5−Trp16 couple are situated close to Pro201 in a β-turn of type VIa (Eriksson et al., 1988). This turn also includes Pro202 which is joined to Pro201 via a peptide bond in the *cis* conformation. It is probable that this turn has to adopt a native conformation before it

can be used as a template for the folding of the N-terminus. Interestingly, Tweedy et al. (1993) have shown that the peptide bond between positions 201 and 202 adopts the *cis* conformation even when Pro202 is replaced with an alanine. Apparently, other parts of the structure put severe constraints on the polypeptide at this turn, leading to a rate-limiting reaction in the folding (Fransson et al., 1992).

Tryptophans in the Core. Studies of a $HCAII_{pwt}$ variant in which the twenty-four N-terminal amino acids were truncated, as well as studies on a W5F/W16F variant (Aronsson et al., 1995), showed that the remaining five tryptophans regained fluorescence properties, that were virtually identical to those of the native state, within 60 s of refolding. The enzymatic activity of both $HCAII_{pwt}$ and of the truncated variant reappears later in two phases having half-times of 2 and 17 min, which are rates of similar magnitudes as those of phases 4 and 5 of the intrinsic fluorescence. Thus, the final very slow rearrangements of the protein structure, to give an active enzyme, do apparently not involve any substantial changes in the environments of tryptophans 97, 123, 192, or 209.

Phases 2 and 3 observed for $HCAII_{pwt}$ describe a loss of fluorescence emission. The analysis shows that these phases contain fluorescence components from tryptophans 123, 192, 209, and 245. Inspection of Table 1 reveals that the contributions from these tryptophans all have the same sign, showing a lowering of the fluorescence emission, which indicates that the two phases describe the folding of two populations of the protein taking different folding pathways. Thus, phases 2 and 3 do not represent sequential folding steps. The rate constant, k_2, is very similar for these four tryptophans, which shows that the formation of structures giving them native-like fluorescence properties is controlled by the same rate-limiting reaction in this population. Notably, k_3 varies by a factor of 2 between the tryptophans 123, 209, and 245, which may indicate that the situation is more complicated for this population. Tryptophans 5, 16, and 97 do not contribute at all to phase 2 or 3.

Trp123 does not emit any light in the native state due to quenching by the neighboring Arg89 and possibly Asp72 which forms a hydrogen bond to the NH of the indole ring (Mårtensson et al., 1995) (Figure 4C). Since Trp123 has obtained native-like fluorescence properties after completion of the third kinetic phase, we propose that its structural environment, at that time, closely resembles the native state including van der Waals contact with Arg89 or Asp72. Trp123 and Arg89 are situated on β-strands 5 and 4, respectively, while Asp72 is the second residue from the C-terminal end of β-strand 3 (Figure 4C). The secondary structure may form very early at these β-strands since they contain several of the residues in the large hydrophobic cluster and constitute the most stable part of the protein (Mårtensson et al., 1993; Svensson et al., 1995). It seems as if tertiary structure forms more rapidly in the region of stable β-structure than in peripheral parts of the protein.

Trp192. The fluorescence of Trp192 decays in a single exponential described by phase 2, which indicates that formation of the structure at Trp192 is controlled by the same rate-limiting reaction regardless of which folding population this tryptophan is situated in. According to our earlier assignments, Trp192 will transfer all its fluorescence to Trp209 in the native state, which in turn is fully quenched by neighboring histidine side chains or by the active site Zn^{2+}−histidine complex (Figure 4D). The kinetic data indicate that the fluorescence from Trp209 becomes quenched in phases 2 and 3 and that Trp192 transfers its light energy to Trp209. Because the energy transfer is distance and angle dependent, it is probable that Trp192 is in a roughly correct position, but its fluorescence properties do not allow a more detailed structural interpretation.

Trp209. The results on Trp209 can be interpreted in much the same manner as for Trp123, indicating that the structure at Trp209 is well-defined and native-like when the third phase is completed. Trp209 is situated on β-strand 7, and its putative quenchers His107 and His119 reside in a loop and on β-strand 5, respectively, while the two Zn^{2+} ligands His94 and His96 reside on β-strand 4 (Figure 4D). The result indicates that much of this structure is formed within 60 s. Earlier studies by Bergenhem et al. (1989) on metal binding to the active site of bovine carbonic anhydrase II showed that a high affinity metal binding is formed within 10 s of folding. However, the tetrahedral coordination leading to an active enzyme is not formed until much later, with a half-time of 9 min under their experimental conditions. Apparently, these late rearrangements of metal coordination seems to be subtle enough not to affect the nearby Trp209.

Equilibrium Unfolding Intermediates and Kinetic Intermediates. We have earlier characterized an equilibrium folding intermediate that is highly populated at 1.3−1.5 M GuHCl (Mårtensson et al., 1993, 1995; Svensson et al., 1995). The results of an experiment in which $HCAII_{pwt}$ is allowed to refold at 1.3 M GuHCl are shown in Figure 2. The rates differ from those observed when refolding at 0.3 M GuHCl, because the conditions are different. Initially, the pattern is similar with a large burst phase followed by a somewhat slower phase of increasing fluorescence. Thereafter, however, the similarities stop, and the fluorescence of $HCAII_{pwt}$ in the experiment at 1.3 M GuHCl levels off, after a minor decrease, at a fluorescence level of 0.95. This fluorescence level is close to the start level of phase 2 in the experiment at the native conditions of 0.3 M GuHCl.

These results seem to indicate that the kinetic intermediate that is observed after approximately 1 s of refolding under native conditions has some properties in common with the equilibrium intermediate. The specific quenching of tryptophan fluorescence by neighboring amino acids is weak or absent in the equilibrium intermediate (Mårtensson et al., 1995), and it is absent in the kinetic intermediate as well. Thus, it seems as if little or no tertiary structure is formed in either of these intermediates.

Both types of intermediates contain a stable and rather compact core that includes a hydrophobic cluster. CD measurements indicate that the equilibrium intermediate contains much of the native β-structure (Mårtensson et al., 1993). The kinetic experiments in the present study give no direct information on the formation of secondary structure. We observe that the tertiary structure is formed earlier at tryptophans that are closely associated with the central β-sheet (i.e., Trp123 and Trp209) than for tryptophans in peripheral parts (i.e., Trp5 and Trp16). Thus, since the tertiary structure at Trp123 and Trp209 is formed in phases 2 and 3, the central β-strands might possibly be present in the kinetic intermediate.

REFERENCES

Aronsson, G., Mårtensson, L.-G., Carlsson, U., & Jonsson, B.-H. (1995) *Biochemistry 34*, 2153−2162.

Bergenhem, N., & Carlsson, U. (1989) *Biochim. Biophys. Acta 998*, 277−285.

Eriksson, A. E., Jones, T. A., & Liljas, A. (1988) *Proteins: Struct., Funct., Genet. 4*, 274−282.

Fransson, C., Freskgård, P.-O., Herbertsson, H., Johansson, Å., Jonasson, P., Mårtensson, L.-G., Svensson, M., Jonsson, B.-H., & Carlsson, U. (1992) *FEBS Lett. 296*, 90−94.

Freskgård, P.-O., Mårtensson, L.-G., Jonasson, P., Jonsson, B.-H., & Carlsson, U. (1994) *Biochemistry 33*, 14281−14288.

Håkansson, K., Carlsson, M., Svensson, L. A., & Liljas, A. (1992) *J. Mol. Biol. 227*, 1192−1204.

Ikai, A., Tanaka, S., & Noda, H. (1978) *Arch. Biochem. Biophys. 190*, 39−45.

Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

Liljas, A., Håkansson, K., Jonsson, B.-H., & Yafeng, X. (1994) *Eur. J. Biochem. 219*, 1−10.

Mårtensson, L.-G., Jonsson, B.-H., Freskgård, P.-O., Kihlgren, A., Svensson, M., & Carlsson, U. (1993) *Biochemistry 32*, 224−231.

Mårtensson, L.-G., Jonasson, P., Freskgård, P.-O., Svensson, M., Carlsson, U., & Jonsson, B.-H. (1995) *Biochemistry 34*, 1011−1021.

Nozaki, Y. (1972) *Methods Enzymol. 26*, 43−50.

Ptitsyn, O. B. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 342−300, W. H. Freeman and Co., New York.

Svensson, M., Jonasson, P., Freskgård, P.-O., Jonsson, B.-H., Lindgren, M., Mårtensson, L.-G., Gentile, M., Borén, K., & Carlsson, U. (1995) *Biochemistry 34*, 8606−8620.

Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J., & Hiromi, K. (1978) *Anal. Biochem. 84*, 370−383.

Tweedy, N. B., Nair, S. K., Paterno, S. A., Fierke, C. A., & Christianson, D. W. (1993) *Biochemistry 32*, 10944−10949.

BI961882A