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Detection and Characterization Using Circular Dichroism and Fluorescence Spectroscopy of a Stable Intermediate Conformation Formed in the Denaturation of Bovine Carbonic Anhydrase with Guanidinium Chloride†

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ABSTRACT: Particularly stable elements of noncovalent structure in bovine carbonic anhydrase have been detected and studied. These are present in a highly populated intermediate state formed during denaturation of the enzyme with guanidinium chloride. The intermediate has been detected by analysis of the denaturation profiles, and some of its structural properties have been characterized by CD and fluorescence spectroscopy, including fluorescence polarization and lifetime measurements. Measurements have been made on the Zn^{2+}–enzyme, Co^{2+}–enzyme, and apoenzyme to ascertain the structural effects of the active-site Zn^{2+}. Kinetic measurements indicate that this intermediate is on the folding pathway from the random coil to the native state.

Of special interest in understanding the factors that determine the three-dimensional structure of a globular protein is the detection and identification of especially stable elements within the overall structure. One approach to this problem comes in studying equilibrium denaturation of the protein, since any intermediates found in the presence of strong denaturants necessarily contain stable elements of secondary or tertiary structure or both. In this paper, we report a spectroscopic analysis (CD and fluorescence)[1] of the guanidinium chloride denaturation of bovine carbonic anhydrase with the aim of detecting and studying stable elements of enzyme structure.

Bovine carbonic anhydrase is a zinc-containing protein composed of a single polypeptide chain that is folded without disulfide cross-links into a compact, globular structure with

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[1] Abbreviations: GdmCl, guanidinium chloride; CD, circular dichroism; DNSA, (dimethylamino)naphthalenesulfonamide.

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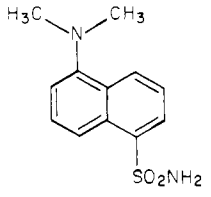
a core of β structure and several α helices (Kannan et al., 1972; Liljas et al., 1972). It has been classified as a parallel α/β protein (Richardson, 1981). The protein is fully unfolded by the action of 3 M GdmCl to yield a random coil (Wong & Tanford, 1973) and refolds upon removal of the denaturing conditions to re-form the native structure (Yazgan & Henkens, 1972; Wong & Tanford, 1973). The zinc remains tightly bound to the fully denatured protein (R.W. Henkens, A. M. Schrag, P. J. Stein, unpublished results) and facilitates the folding process (Yazgan & Henkens, 1972; Wong & Hamlin, 1975; Ikai et al., 1978). Yazgan & Henkens (1972) were the first to detect kinetic intermediates in the folding of carbonic anhydrase, although they did not detect stable intermediates from their initial measurements of denaturation profiles. Wong & Tanford (1973) observed intermediates in both kinetic and equilibrium measurements and concluded from the latter measurements that carbonic anhydrase denaturation by GdmCl is a thermodynamically reversible process occurring in separate stages. But Ikai et al. (1978) demonstrated that the native structure is not regenerated from the intermediate stage of unfolding near 2.0 M GdmCl. Thus, the denaturation process is more complex than originally envisioned and appears to be a thermodynamically irreversible process (Ikai et al., 1978).

We report here on CD and fluorescence measurements that further characterize the denaturation process of carbonic anhydrase and the structure of the stable intermediate found near 2 M GdmCl. Measurements of fluorescence intensity of an extrinsic active-site probe, DNSA (I), and fluorescence



I

intensity, wavelength, lifetime, and polarization of tryptophan side chains within the protein, as well as CD measurements of the aromatic and backbone structure of the protein, have been used to monitor the unfolding of carbonic anhydrase from the native structure to the random coil. The effect of the Zn^{2+} is also examined through comparative measurements on the Zn^{2+}–enzyme, Co^{2+}–enzyme, and apoenzyme. We show that (1) a stable intermediate is the predominant form present with 2.0 M GdmCl and that this intermediate contains major elements of secondary and tertiary structure, (2) Zn^{2+} stabilizes the structure of both the native and intermediate conformations, and (3) the intermediate can refold to the native structure when the denaturant is removed, with a rate that is the same as the rate-limiting step in the overall folding process from the random coil to the native state.

Experimental Procedures

Materials. Bovine carbonic anhydrase obtained from Worthington Biochemical Corp. was purified further by chromatography on DEAE-Sephadex (Yeagle et al., 1975) or by affinity chromatography using *p*-(aminomethyl)benzenesulfonamide coupled to the carboxyl groups of an agarose ion exchange (Khalifah et al., 1977). Protein concentrations were determined from the absorbance of the solution ($\epsilon_{280nm} = 5.7 \times 10^4$ M^{-1} cm^{-1}; Lindskog & Nyman, 1964). The apoenzyme was prepared as described by Lindskog & Malmstrom (1962). Enzymatic activity of the inactive apoenzyme was fully recovered on addition of zinc ion. Cobalt(II)-substituted enzyme was prepared by dialysis of the apoenzyme against 1.0×10^{-2} M $CoCl_2$ or by direct addition of 1 equiv of $CoCl_2$ (cf. Yeagle et al., 1975), followed in both cases by dialysis against water.

Guanidine hydrochloride from Schwarz/Mann (mp 184.5–185.5 °C) was used without further purification. A 6.0 M solution had no significant fluorescence or absorption above 250 nm. Concentrations of GdmCl were determined from refractive index (Nozaki, 1972).

(Dimethylamino)naphthalenesulfonamide, DNSA, was prepared as described (Weber, 1952).

Methods. Fluorescence was measured with a Perkin-Elmer MPF-3 spectrofluorometer equipped with an R446 photomultiplier. On this instrument, emission of L-tryptophan in the region 320–400 nm deviated very little from the published corrected spectrum (Teale & Weber, 1957) so that correction was unnecessary. Quantum yields relative to L-tryptophan were calculated by using $Q = 0.12$ for aqueous L-tryptophan solutions (Chen, 1973), which were always freshly prepared. Samples were thermostated, and measurements were made in the concentration range where fluorescence is directly proportional to concentration.

Generally, denaturation was carried out in solutions to which NaCl was added to maintain a constant ionic strength of 3.0 M (GdmCl and NaCl), except as otherwise noted. In this way, the fluorescence properties are less influenced by general salt effects or specific effects of chloride ion and more clearly reflect conformational changes. Before equilibrium measurements, samples were allowed to equilibrate for at least 12 h.

Fluorescence lifetime measurements were performed by the phase-modulation method (Spencer & Weber, 1969) on an SLM-4800A subnanosecond spectrofluorometer. The effects of rotational diffusion on the observed lifetimes were eliminated by exciting with vertically polarized light and observing time emission through a polarizer oriented at the "magic angle" (Spencer & Weber, 1970). The emission of the protein was observed through a UG1 broad band filter with maximum transmission at 360 nm (Klinger Scientific Corp.) or through a monochromator at 344 nm with 8-nm band-pass. All lifetime measurements were made relative to a glycogen-scatter solution (Spencer & Weber, 1969). Protein concentrations were varied from 5 to 10 μM for all measurements. Since the time response of the photomultiplier tube is known to be dependent upon wavelength (Muller et al., 1965), *p*-terphenyl (Lakowicz & Weber, 1973) was measured under the same conditions as the protein, and the expected 0.9-ns lifetime (Lakowicz & Cherek, 1980) was obtained. Therefore, no corrections were made to the lifetimes measured for the protein.

Polarization measurements were also made on the SLM-4800A spectrofluorometer in a T format so that the horizontal and vertical components of the emission could be observed simultaneously (Weber & Bablouzian, 1966) or with the Perkin-Elmer MPF-3 spectrofluorometer.

CD measurements were made on a Jasco J-500A spectropolarimeter equipped with a Jasco DP-500 data processer. Far-Ultraviolet spectra of 2 μM carbonic anhydrase were obtained in 2-mm path-length cells. Near-ultraviolet spectra of 25 μM protein were obtained in 1-cm path-length cells. All spectra reported were averages of four to eight individual spectra and were corrected for base-line contributions. The cell compartment temperature was maintained at 25 °C.

Results

Transition from Native to Denatured State. Figure 1 summarizes changes in fluorescence properties that occur on unfolding of the apoenzyme, Co^{2+}–enzyme, and Zn^{2+}–enzyme. For all three forms, the transition from the native to the de-

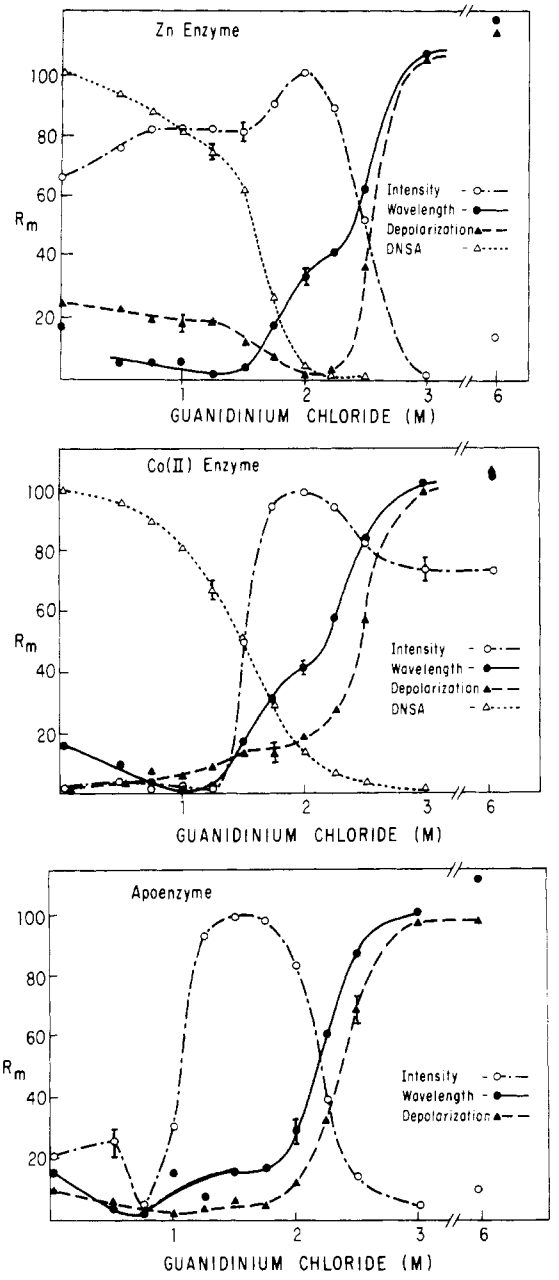


FIGURE 1: Denaturation transitions of Zn^{2+}–carbonic anhydrase, Co^{2+}–carbonic anhydrase, and apocarbonic anhydrase determined from changes in intrinsic fluorescence properties (emission intensity, wavelength, and depolarization) and changes in the fluorescence emission of an active-site probe, dansylsulfonamide (DNSA). Excitation was at 280 nm for both the probe fluorescence and intrinsic fluoresence. Emission was measured at 456 nm for the probe and 338 nm for the intrinsic fluorescence. Samples were maintained at 24 °C, pH 7.5, in a Tris-HCl buffer in which NaCl was added to maintain a constant ionic strength of 3.0 M NaCl plus GdmCl up to 3.0 M GdmCl. Note the break in the data at 3.0 M GdmCl. R_m is the relative change in the particular fluorescence parameter based on the maximum and minimum values of that parameter observed between 0 and 3 M GdmCl. The error bars give the standard errors of the measurements.

natured state occurred in at least two separate stages. This can be determined by inspection of the denaturation profiles (Figure 1). The stages are characterized by sigmoidal increases or decreases in a particular fluorescence property. The emission wavelength increased in both stages, the emission intensity increased in the first stage and then decreased in the second, and the depolarization decreased in the first stage (for the Zn^{2+}–enzyme) and then increased in the second (Figure 1). The major changes in polarization occurred in the second

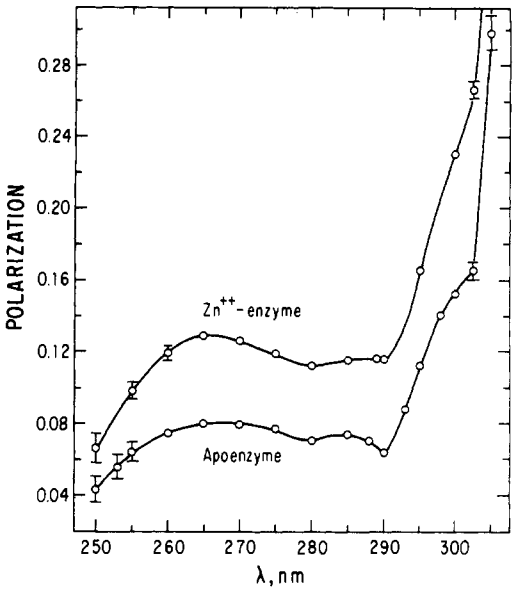


FIGURE 2: Polarized fluorescence–excitation spectra of Zn^{2+}–carbonic anhydrase and apocarbonic anhydrase in 2.0 M GdmCl. Polarized emission measured at 344 nm with an 8-nm band-pass on the SLM-4800 spectrofluorometer (see Methods). Samples were maintained at 5 °C, pH 7.5, in a 0.1 M Tris-HCl buffer.

stage. The first stage was complete in 2.0 M GdmCl, and the second was complete in 3.0 M GdmCl (Figure 1). The first stage of denaturation was strongly influenced by the presence of Zn^{2+} or Co^{2+} [cf. the midpoint of the transitions for the apoenzyme (1.1 M GdmCl), Co^{2+}–enzyme (1.5 M GdmCl), and Zn^{2+}–enzyme (1.8 M GdmCl)] (Figure 1). The metal had a smaller but significant effect on the second stage of the unfolding reaction. The stability toward unfolding in both stages was Zn^{2+}–enzyme > Co^{2+}–enzyme > apoenzyme. Figure 1 also shows transition curves determined by following the fluorescence intensity of DNSA, a specific inhibitor that binds in the active-site crevice (Chen & Kernohan, 1967).

Intermediate State in 2.0 M GdmCl. The fluorescence polarization spectrum of the intermediate state in 2.0 M GdmCl is shown in Figure 2. The spectrum is characterized by a broad maximum near 270 nm, a sharp band at 288 nm, and a region of rapidly increasing polarization at longer wavelengths. It is similar to that observed for tryptophan or its simple derivatives in a highly viscous medium (Lynn & Fasman, 1968) and to the polarization spectra observed for intact proteins (Weber, 1961; Konev, 1967; Kitchell et al., 1977). In particular, the ratio of polarization values for carbonic anhydrase at 305 and 270 nm, $P_{305nm}/P_{270nm} = 2.1$, and the maximum observed polarization, $P_{310nm} = 0.37$, are within the range of values reported by Weber (1961) and are also comparable to the value observed for free tryptophan in rigid solutions at low temperature (Weber, 1961).

The CD spectra for three states of bovine carbonic anhydrase, the native, intermediate, and random coil, are shown in Figure 3. The far-ultraviolet spectra, 200–240 nm (left side of Figure 3), are due largely to the peptide group, although there may be some contribution from the aromatic groups. We have used these data to estimate the fractions of helix, β structure, and unordered coil in the intermediate state, using protein reference spectra and the relationship (Chen et al., 1974; Yang, 1976)

$$[\theta] = f_H[\theta]_H + f_\beta[\theta]_\beta + f_R[\theta]_R$$

First, the fraction helix was calculated by using $[\theta]$ at 224 nm, where the contributions from β structure and unordered

Table I: Fluorescence of Bovine Carbonic Anhydrase at 25 °C

state	intensity[a]	wavelength (nm)	depolarization	lifetime[b] (ns)	quantum yield[c]	DNSA (intensity)
		Zn^{2+}-Carbonic Anhydrase				
native (0 M GdmCl)	63	338	13.1	4.0	0.072	90
intermediate (2.0 M GdmCl)	70	341	10.6	3.5	0.063	5
random coil (3.0 M GdmCl)	50	348	20.4	2.7	0.050	0
		Co^{2+}-Carbonic Anhydrase				
native (0 M GdmCl)	30	337	9.3	2.2	0.040	33
intermediate (2.0 M GdmCl)	58	340	11.3	2.5	0.045	5
random coil (3.0 M GdmCl)	50	349	21.5	2.7	0.050	1
		Apocarbonic Anhydrase				
native (0 M GdmCl)	51	336	10.3	3.6	0.064	
intermediate (1.6 M GdmCl)	83	336	9.8			
random coil (3.0 M GdmCl)	50	348	22.4	2.7	0.050	

[a] Fluorescence amplitude at the emission maximum in arbitrary units relative to the random coil taken as 50 units. Excitation at 280 nm. [b] Excitation at 288 nm. Measurements at 34 °C. [c] Calculated from the quantum yield of the native zinc-enzyme and the observed lifetime relative to the zinc-enzyme.

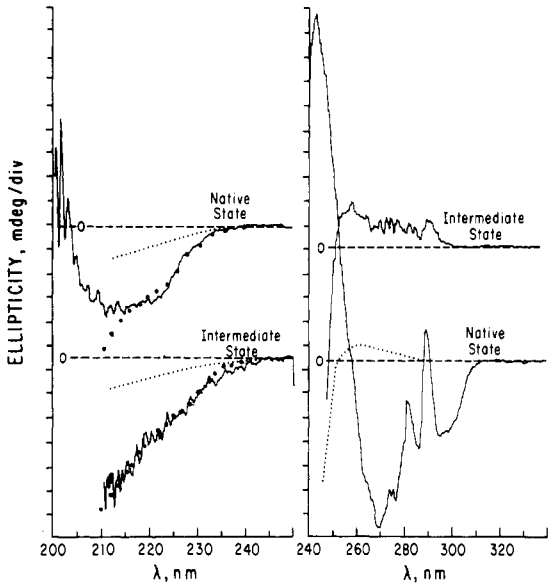


FIGURE 3: Far-ultraviolet and near-ultraviolet CD of the native state of carbonic anhydrase in 0.1 M Tris-HCl buffer, pH 7.5, and of the intermediate state in the same buffer containing 2.0 M GdmCl. The sample concentrations and path lengths are given under Experimental Procedures. The spectra of the native and intermediate states are offset vertically, and the zero line is indicated by dashes. The CD spectrum of the random coil in 3 M GdmCl (dotted line above the spectra of the native and intermediate states) is included for comparison. The percent helix, β structure, and unordered coil was estimated by fitting the far-ultraviolet spectra (left side of figure) to reference spectra as described in the text; the points plotted over the actual far-ultraviolet CD spectra indicate the fitted CD curves for the native and intermediate states.

coil forms are negligible. Then, when this value for the fraction helix was used, the best fit to the experimental curve was then found by minimizing the variance from 210 to 240 nm between the experimental curve and a combination of CD curves for helix, β structure, and unordered forms.

The results of this analysis are shown in Figure 3. The calculated CD curve is indicated by the points. The fit to the experimental CD is good from 210 to 240 nm. The fractions of helix, β structure, and unordered form of the intermediate estimated from the analysis are $f_H = 0.1$, $f_\beta = 0.25$, and $f_C = 0.65$.

A similar analysis was used for the native structure. The best fit to the experimental CD is not as good, especially from 210 to 215 nm. The best values are $f_H = 0.08$, $f_\beta = 0.25$, and $f_C = 0.67$.

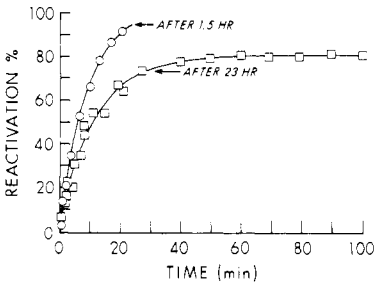


FIGURE 4: Course of recovery of the *p*-nitrophenyl activity of carbonic anhydrase on refolding from the intermediate to the native state. The refolding reaction was initiated, after 1.5 or 23 h, by a dilution from 2.0 to 0.7 M GdmCl. The percentage reactivation during the course of the refolding reaction was determined as described in the text.

The near-ultraviolet CD of the intermediate contains small positive bands in the region between 250 and 300 nm that are due to aromatic chromophores in asymmetric environments. These aromatic Cotton effects are smaller and less complex than those of the native structure (Figure 3).

Table I compares the fluorescence parameters, including fluorescence excited-state lifetimes, for the native, intermediate, and denatured states of the Zn^{2+}–enzyme, Co^{2+}–enzyme, and apoenzyme.

Regeneration of the Native State. The kinetics of reactivation was measured to test for reversibility and to obtain information on whether the intermediate state found in 2.0 M GdmCl is on the pathway of folding from the random coil to the native state. Two representative kinetic curves for reactivation are shown in Figure 4.

These curves were obtained by using *p*-nitrophenyl acetate as substrate to measure the regeneration of the native, enzymatically active state. The method is a slight modification of that used previously (Ko et al., 1977). A small volume (10 μL) of substrate solution (dissolved in acetone) was added to the reaction mixture, and the increase in absorbance at 400 nm due to substrate hydrolysis was monitored during the course of the refolding reaction. The extent of reactivation at intervals during the refolding reaction was calculated from the slope of the curve, which was divided by the slope of the corresponding curve for the native enzyme to obtain the percent reactivation.

As shown in Figure 4, regeneration of the native state occurred with a half-life of 7 min; 80% of the enzymatic activity was recovered even after exposure to the denaturing conditions for 23 h. Shorter periods resulted in better recovery of the

activity (Figure 4), and longer periods resulted in the progressive decrease in recovery of the activity (not shown). Reactivation kinetics occurred with a good fit to the calculated curve (solid lines in Figure 4) for a first-order process.

Discussion

Unlike a number of other larger proteins, which contain several structural domains, carbonic anhydrase appears to be folded in a single unit that includes the entire protein. The only domainlike feature is composed of strands 2–6 of β structure (Kannan et al., 1972). Ordinarily, it would be expected that such a protein would unfold in a single cooperative transition in which all intermediate conformations would be at most only marginally stable. But the protein actually unfolds in a stepwise manner, indicating that there is at least one intermediate state that is sufficiently stable to persist in high concentrations of GdmCl.

Sensitized DNSA emission was used to probe the formation of the intermediate state (Figure 1). Comparison of the various transition curves (Figure 1) shows that the bound DNSA emission disappears on formation of the intermediate state and that the probe has little if any effect on the position of the structural transition. DNSA bound near the center of the enzyme is a particularly good acceptor for the resonance transfer of energy from tryptophan residues within the native enzyme molecule (Chen & Kernohan, 1967), and excitation of tryptophan residues at 280 nm results in efficient energy transfer to DNSA with resulting emission at 456 nm. The disappearance of this sensitized DNSA emission indicates that the intermediate state of the Zn^{2+}–enzyme is fully formed near 2.0 M GdmCl (Figure 1) and that the DNSA does not remain bound to the intermediate structure.

Contributions from aromatic amino acid side chains to the near-ultraviolet spectroscopic properties were used to probe the tertiary structure. These were especially useful in studying changes in the environment of tryptophan side chains. For the Zn–enzyme, the structural changes associated with complete GdmCl denaturation result in a 10-nm red shift in emission, a 30% decrease in quantum yield, about a 60% increase in depolarization, and almost complete loss of the multiple Cotton effects in the 240–310-nm region of the CD.

Bovine carbonic anhydrase contains seven tryptophan residues. All of these, except Trp-243, are conserved in bovine and both human forms of the enzyme (Sciaky et al., 1974). At least four (Kannan et al., 1972) and possibly as many as six (Yazgan & Henkens, 1972) of the tryptophan residues are located in the interior of the structure. Trp-4 and Trp-14 in human carbonic anhydrases are situated in a pronounced aromatic cluster (aromatic cluster I) located on the top side of the large β sheet close to the active site (Liljas, 1971). Trp-207 is next to the active-site metal. Six phenylalanine residues (aromatic cluster II) are located in a large hydrophobic core on the other side of the large β sheet (Liljas, 1971).

The near-ultraviolet spectroscopic properties of the intermediate structural state in 2.0 M GdmCl suggest a generally compact tertiary structure. For example, the near-ultraviolet difference spectrum indicates that the tryptophan residues are still buried in the structure (Yazgan & Henkens, 1972); the low fluorescence depolarization, which is comparable to that of the native state, suggests that there is still no major increase in internal motion of the tryptophan residues.

The fluorescence quenching caused by Co(II) (Table I) can mean that Trp-207 is still in close proximity to the metal. The quenching is attributed to an energy relay system (Stein, 1976), in which excited-state energy is relayed via resonance transfer from outlying tryptophan residues to Trp-207, which then rapidly loses its excited-state energy by direct physical interaction with the paramagnetic Co(II) ion. Such a process would be very sensitive to the proximity of Trp-207 and the metal ion. It would also be sensitive to the distance between tryptophans involved in the relay system. The substantial quenching observed when Co(II) replaces Zn(II) in the intermediate state as well as the native state (Table I) suggests that Trp-207 is still in contact with the metal.

On the other hand, the shift in emission wavelength indicates a change in the environment of some tryptophan residues, and the loss of negative CD bands between 290 and 310 nm, which must originate in tryptophan side chains since no other amino acid residue has bands at these wavelengths, suggests changes specifically in aromatic cluster I, which presumably gives rise to this feature in the CD spectrum (Beychok et al., 1966). Possibly, there are alterations in aromatic cluster II as well, which could cause some of the other CD changes in the 250–270-nm region where phenylalanine side chains can contribute (Romans, 1973).

The strong contributions of the aromatic chromophores to the CD have complicated estimates of helix and β structure of carbonic anhydrase. As can be seen in Figure 3, the fit to standard CD spectra is not very good, and the estimates of helix and β content are somewhat low—from the X-ray analysis (Kannan et al., 1972), about 37% of the residues are in β structure and about 20% are within helices (although only a few residues belong to the classical α-helix type).

The contributions of aromatic residues to the CD is much less in the intermediate state, so presumably the estimates of secondary structure are more accurate here. Furthermore, the helical segments could be more regular, which would result in more normal CD curves and a better agreement of the CD with reference spectra. In any case, there is a better fit between experimental and predicted CD curves. On the basis of the CD, the intermediate contains about 25% of the residues in β structure and about 10% within helices. This amount of β structure is compatible with eight strands of β sheet remaining intact.

Stabilization of the intermediate by metal indicates that the metal is still bound, possibly to a domainlike structural element involving a part of the major β sheet and some additional elements of the chain. The Zn^{2+} binding ligands are on strands 4 and 5, while the top of the active site involves the amino terminus and the large loop connecting strands 8 and 9, so it seems reasonable that the Zn^{2+} would stay bound but the DNSA come off. But it seems unlikely that an isolated β sheet would be stable without anything covering at least the bottom side. This could be accomplished by the involvement of some additional loops of the chain, such as the connections from strands 1–2, 6–7, and 7–8.

Equilibrium measurements, such as those reported in Figure 1, can detect only energetically stable conformations—they do not provide information on the pathway between the denatured and native states. On the other hand, kinetic measurements follow the process with the lowest activation energy and detect intermediates on the folding pathway. But in some cases energetically stable conformations detected in equilibrium measurements are the same as intermediates on the folding pathway. This may be the situation for the stable intermediate conformation formed in the denaturation of carbonic anhydrase with GdmCl. The half-life for reactivation from the intermediate state in 2.0 M GdmCl is 7 min. This time is comparable to that observed for the reactivation on refolding from the fully unfolded state (Ko et al., 1977; Stein & Henkens, 1978), suggesting that both processes have the same

rate-limiting step (Ikai et al., 1978) and that the stable intermediate described in this work is the same as the kinetic intermediate we have observed previously in the folding process from the random coil to the native state (Yazgan & Henkens, 1972; Ko et al., 1977; Stein & Henkens, 1978). This kinetic intermediate is formed rapidly.

On the basis of these results, we suggest that the intermediate conformation consists of a major folding domain, which is formed rapidly in the overall folding process, and that the conversion of this intermediate to the native structure was slow because it involves conformational changes in a compact, relatively immobile intermediate structure.

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