Denaturation of Bovine Carbonic Anhydrase B by Guanidine Hydrochloride

A PROCESS INVOLVING SEPARABLE SEQUENTIAL CONFORMATIONAL TRANSITIONS*

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SUMMARY

The denaturation and renaturation of bovine carbonic anhydrase B is a thermodynamically reversible process, uncomplicated by aggregation or disulfide bond formation. The reaction is less cooperative than is the unfolding and refolding of most globular proteins, in that distinct successive stages can be observed both in equilibrium and kinetic measurements. This enzyme is therefore ideally suited for investigation of the mechanism of folding of an unfolded polypeptide chain.

As part of this work the properties of the native enzyme have been carefully reinvestigated. A somewhat smaller molecular weight (29,000) and sedimentation coefficient ($s_0 = 2.89$ S) than previously reported were obtained. The absence of cystine or cysteine has been unambiguously established.

EXPERIMENTAL PROCEDURES

Materials

Crude mixtures of bovine carbonic anhydrases were obtained from the Sigma Chemical Co., St. Louis, Mo. (Lots 118B-2060 and 76B-1960), and from Worthington Biochemical Corp., Freehold, N. J. (Lots CA9HA, CA9HB, CA0CA, CA8HA). Different activity units were given on different lots ranging from 3100 to 4500 Roughton-Booth units per mg for the Sigma samples, and 2100 to 4500 Wilbur-Anderson units per mg for the Worthington samples.

Pure guanidine hydrochloride was obtained by the method of Nozaki and Tanford (1) or from commercial products satisfying the criteria of purity described earlier (2). All other chemicals used were best available reagent grade products.

Methods

Isolation of Pure Protein from Crude Mixture—Pure protein was obtained by chromatography on DEAE-Sephadex, following a procedure similar to that described by Armstrong et al. (3) for purification of the human enzyme.

Esterase Activity—Esterase activity was assayed by the method of Packer and Stone (4), using p-nitrophenyl acetate as a substrate. The measured activity was completely abolished by the specific inhibitor acetazolamide, showing the absence of any contaminating protein with esterase activity.

Amino Acid Analysis—Protein samples (0.5 to 1 mg) were hydrolyzed in 6 N HCl under reduced pressure for 24, 48, and 72 hours at 110°C. The amino acid compositions of the hydrolysates were determined as described by Armstrong et al. (3) for purification of the human enzyme.

Ultracentrifugation—Sedimentation velocity and equilibrium measurements were carried out in a Beckman-Spinco model E ultracentrifuge. The short column, high speed technique of Yphantis (8), employing interference optics, was used for equilibrium measurements. The partial specific volume of the native protein was obtained as follows:

\[
\phi = \frac{1}{3}(1 + 2 \rho_v \rho_o)
\]

where $\rho_v$ is the density of the void and $\rho_o$ is the density of the solution. The total weight-average molecular weight was calculated from the sedimentation coefficient using the equation:

\[
M_w = \frac{s^2}{k} (1 + 2 \rho_v \rho_o)
\]

where $s$ is the sedimentation coefficient and $k$ is the Boltzmann constant.

The final step in protein biosynthesis is the folding of the polypeptide chain into its native three-dimensional structure. It is an intermediate step in making genetic information available for the functioning of the corresponding protein. An approach to understanding this process is to study the kinetics of refolding of proteins which have been transformed to random coiled by the action of guanidine hydrochloride. It is desirable for an initial study to use a protein containing no cysteine or cystine, so as to focus exclusively on the successive conformational changes without complications from sulphydryl group oxidation. An additional requirement is that the denaturation by guanidine hydrochloride should not be a two-state process, i.e. that intermediate conformational states be experimentally detectable. It will be shown in this paper that bovine carbonic anhydrase B satisfies these requirements. Denaturation by guanidine hydrochloride will be shown to be a reversible process, separable into at least three stages by equilibrium measurements as well as by preliminary kinetic data.

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enzyme was calculated from the amino acid composition by the procedure of Cohn and Edsall (9). The value obtained was $\theta = 0.733$ cc per g.

Intrinsic Viscosity—Viscosity measurements were made using Cannon-Manning semi-microcapillary viscometers equilibrated in a water bath thermostated at 25.00 ± 0.005°C. Prior to measurement protein solutions were dialyzed against appropriate solvents at least 24 hours for 0.1 M NaCl and 4 days for 6 M GuHCl. Generally five to eight flow time measurements were made on each solution.

Gel Filtration Chromatography in 6 M GuHCl—The procedure of Fish et al. (10) was followed, using 6% agarose gel. The agarose column was calibrated with several reduced and carboxymethylated proteins of known molecular weight.

Disc Gel Electrophoresis—The method described by Ornstein (11) was followed for disc gel electrophoresis at pH 8.9. Electrophoresis at pH 3.5 and in urea-acetic acid was performed according to the procedure of Panyim and Chalkley (12).

Ultraviolet Difference Spectra—These spectra were obtained at 25 ± 1°C with a Cary 15 double beam spectrophotometer. Either the 1.0-cm rectangular cell or the tandem double cell of Herskovits and Lasokowski (13) was employed. In obtaining the difference spectra, a scan was first obtained with the reference solution in both the sample and the reference cells placed in their respective compartments. This is the zero base line for calculation and is necessitated by the separate detectors design of this spectrophotometer (14). The sample was scanned, and the positions of the cells were then exchanged to obtain another spectrum.

Optical Rotatory Dispersion—Optical rotatory dispersion measurements were made in a Cary model 60 recording spectropolarimeter. The temperature of the sample cell was approximately 25 ± 1°C and no difference was observed between results obtained with the cell thermostated at 25°C and those obtained at this temperature. No significant stray light effect was observed (15) and no band width dependence of the rotations was detected. Results are expressed in terms of reduced mean residue rotation [°]. The refractive index of GuHCl solutions was calculated as described by Hooker (16). A mean residue weight of 114 was calculated from the amino acid analysis.

Circular Dichroism—CD measurements were made in the same instrument, equipped with a model 6001 CD attachment. Results are expressed in terms of molar ellipticity, [θ].

Equilibrium Measurements—Protein stock solution was added by weight to GuHCl solutions of known concentration, such that the final protein concentration was between 0.06 and 0.25 mg per ml. Attainment of equilibrium was established by leaving the solutions at room temperature for about 10 to 14 hours. Thermodynamic equilibrium was established by dilution of protein solutions that had been allowed to stand for one hour in the denatured state in aqueous GuHCl.

Complete Renaturation—The denatured protein can be renatured by removing GuHCl. This is done either by exhaustive dialysis of the denatured protein against a large volume of 0.1 M NaCl solution at pH 6 to 7 in the cold (−4°C) or by dilution of the denatured protein to a final GuHCl concentration of 0.7 M or higher, followed by exhaustive dialysis. In the latter procedure, precipitation of the protein frequently occurred if the concentration of GuHCl before dialysis was 0.5 M or below.

Kinetic Measurements—Preliminary kinetics experiments of unfolding and refolding were conducted with the Cary 15 spectrophotometer. Complete, rapid mixing within a few seconds (4 to 7 s) was achieved by adding the protein sample on a drilled Teflon plunger. The presence of a very low concentration of GuHCl in the diluent prevents precipitation of protein in most refolding experiments.

Optical Rotatory Dispersion—Optical rotatory dispersion measurements were made with a Perkin-Elmer model 303 automatic absorption spectrophotometer. The DTNB method of Ellman (17) was employed to determine the −SH content both in 0.1 M NaCl and in 6 M GuHCl.

RESULTS

Purification—The conditions for purification on DEAE-Sephadex, together with a typical elution pattern, are shown in Fig. 1. A small peak (I) emerges from the column at the starting condition. The protein in this fraction has a very low esterase activity, about 5 to 10% of the major carbonic anhydrase component. The buffer is then changed to 0.05 M Tris at the same pH. A large broad peak (II) with a specific activity of 64 min−1 was eluted. The last two proteins were eluted by a 0.87, 0.1 M Tris buffer containing 0.1 M NaCl. The major peak (III) is a green protein with a specific activity (35 min−1) about 50% of the major carbonic anhydrase peak. The last protein fraction is shown as a large shoulder (IV) of the green protein peak and consists of a brown protein which is presumably denatured hemoglobin, since the characteristic heme absorption at 415 nm was observed in the ultraviolet spectrum.

The carbonic anhydrase fraction II as isolated by this procedure was found to be a single pure component by disc gel electrophoresis at pH 9, as shown in Gel B of Fig. 2. A similar result was obtained by electrophoresis at pH 3.5 in urea-acetic acid (Gel C in Fig. 2). In sedimentation velocity experiments a single symmetrical peak was observed at all initial concentrations (1 to 10 mg per ml) and for the entire passage of the protein through the ultracentrifuge cell.

Fig. 1. Elution of bovine carbonic anhydrases on DEAE-Sephadex A-50 Column chromatography. The starting material was a commercial chloroform-ethanol extract of bovine erythrocytes. The eluting buffers were 0.02 M Tris for Peak I, 0.05 M Tris for Peak II, and 0.1 M Tris plus 0.5 M NaCl for Peaks III and IV; all at pH 8.7. Approximately 15 ml were collected for each fraction at a flow rate of about 60 ml per hour. All procedures were carried out in the cold room (5°C). Peak II (bovine carbonic anhydrase B, BCAB) is the purified enzyme B.
Isoleucine. Methionine. Valine. Leucine. Phenylalanine. Tyrosine. Tryptophan. Total.

Alanine. Histidine. Glutamic acid. Threonine. Aspartic acid. Half-cystine. Glycine. Proline. Serine. Arginine.

was found to be 1 mole per mole of protein. The extinction coefficient of the protein for a 1 mg per ml solution in a 1-cm cell was found to be 1.83 at 280 nm. The zinc content, as measured by atomic absorption spectrophotometry, was found to be 1 mole per mole of protein.

**Table I**

| Amino acid    | Residues per molecule* after hydrolysis for | Nyman and Lindskog, 1964 (20) |
|---------------|---------------------------------------------|-------------------------------|
|               | 24 hrs | 48 hrs | 72 hrs | Average |
| Lysine        | 19.4   | 19.5   | 16.6   | 18.5    | 18.7 |
| Histidine     | 10.8   | 10.9   | 11.0   | 10.9    | 11.3 |
| Arginine      | 0.5    | 0.8    | 0.9    | 0.4     | 0.9  |
| Aspartic acid | 31.5   | 30.9   | 31.9   | 31.4    | 32.1 |
| Threonine     | 14.2   | 13.8   | 14.6   | 14.2    | 14.5 |
| Serine        | 16.0   | 14.8   | 14.8   | 16.4    | 16.1 |
| Glutamic acid | 23.9   | 23.3   | 24.4   | 23.9    | 23.7 |
| Proline       | 19.6   | 19.8   | 22.1   | 20.5    | 19.5 |
| Glycine       | 10.9   | 19.8   | 21.0   | 20.2    | 20.2 |
| Alanine       | 17.3   | 16.9   | 15.7   | 16.6    | 16.9 |
| Half-cystine  | 0.0    | 0.0    | 0.0    | <0.2    |
| Valine        | 16.1   | 17.4   | 18.1   | 19.6    | 19.9 |
| Methionine    | 3.2    | 3.4    | 3.2    | 3.3     | 3.1  |
| Isoleucine    | 4.8    | 5.1    | 4.7    | 4.9     | 5.0  |
| Leucine       | 26.8   | 26.0   | 26.2   | 26.3    | 26.2 |
| Tyrosine      | 7.7    | 7.9    | 7.6    | 7.7     | 7.9  |
| Phenylalanine | 10.9   | 11.2   | 11.0   | 11.0    | 11.3 |
| Tryptophan    | 7.2    | 7.2    | 7.2    | 7.2     | 7.0  |
| Total         | 282.0  | 282.0  | 282.0  | 282.0   | 282.0 |

* Based on a molecular weight of 29,000.
* Extrapolated to zero time of hydrolysis.
* Determined as cysteic acid after performic acid oxidation.
* Extrapolated to maximum value.
* Measured by the spectrophotometric method of Edelhoch (7).

The extinction coefficient of the protein for a 1 mg per ml solution in a 1-cm cell was found to be 1.83 at 280 nm. The zinc content, as measured by atomic absorption spectrophotometry, was found to be 1 mole per mole of protein.

**Amino Acid Composition**—Carbonic anhydrase B from all sources examined so far contains no disulfide bonds, but usually does contain 1 cysteine residue per mole (18). Lindskog and Malmström (19) and Nyman and Lindskog (20) found that bovine carbonic anhydrase B contains no cysteine, but there are indications to the contrary in the work of Scott and Mendive (21), Keller et al. (22), Liedländer and Stegemann (23), and Liedländer (24). Since the absence of cysteine is crucial to the studies we seek to perform, the amino acid composition of carbonic anhydrase as purified for this study was carefully determined, with the results given in Table I. The results agree within the limitations of the method with those reported by Nyman and Lindskog (20), but differ significantly from the earlier analysis of Liedländer and Stegemann (23). The absence of cysteine indicated by our analysis was confirmed by DTNB titration of both native and denatured protein.

**Molecular Weight**—Sedimentation equilibrium data were obtained for the native protein in 0.1 NaCl, pH 7.0, and for the denatured protein in 6 M GuHCl. Plots of the logarithm of fringe displacement versus the square of the radial distance were perfectly linear from a displacement of 50 μ to the bottom of the solution column, attesting to the size homogeneity of the sample. Using the calculated partial specific volume, ν = 0.735 cc per g, a molecular weight of 29,000 was obtained. The same molecular weight was obtained for the denatured protein in 6 M GuHCl if the apparent specific volume, ν′ (25), was placed equal to 0.726 cc per g. This parameter is typically about 0.01 cc per g less than ν as a result of about 5% preferential association with GuHCl (26).

A molecular weight of 29,000 for the denatured polypeptide chain of the enzyme was also obtained by gel chromatography in 6 M GuHCl. No detectable trace of any smaller or bigger component was seen.

**Gross Conformation of Native Enzyme**—The sedimentation coefficients of carbonic anhydrase were measured as a function of protein concentration. Extrapolation of the linear plot to zero protein concentration yields S₂₀,w = 2.89 S. The frictional ratio (27) corresponding to this value is Φ₀ = 1.14. The intrinsic viscosity was measured and found to be 3.7 cc per g. Both hydrodynamic parameters are within the range of typical globular proteins.

**ORD and CD**—The optical rotatory dispersion spectrum of the native protein is shown in Fig. 3. The results are similar to those reported for bovine carbonic anhydrase by Rosenberg (28) and for human carbonic anhydrases by Myers and Edsall (29) and by...

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**Fig. 2.** Disc gel electrophoresis pattern of carbonic anhydrase. Gels A and B were run with the pH 9 buffer system for the starting material and purified enzyme B, respectively. Gel C was in the urea-acetic acid system for the purified enzyme B.

**Fig. 3.** Optical rotatory dispersion curves for native carbonic anhydrase B in 0.1 NaCl (solid curve) and denatured carbonic anhydrase in 6 M GuHCl, 0.1 NaCl (dotted curve); both at pH 7.0 and were measured at 25°. Inset, ORD curves at lower wave length, conditions are the same.
FIG. 4. Circular dichroism spectra for native carbonic anhydrase B (solid curve), and denatured carbonic anhydrase (dotted curve) from 255 to 320 nm.

FIG. 5. Ultraviolet difference spectrum of carbonic anhydrase B in 6 M GuHCl, 0.1 M NaCl, and at pH 7.0.

Beychok et al. (30), but the lower portion between 285 and 260 nm is in slight disagreement with Rosenberg's data. The circular dichroism spectrum in the aromatic absorption region is shown in Fig. 4. It differs significantly from the circular dichroism spectra of bovine carbonic anhydrases as reported by McFarland and Coleman (31).

Characterization of Product of Denaturation—The product of denaturation by guanidine hydrochloride is a single randomly coiled polypeptide chain by all criteria. The intrinsic viscosity in 6 M GuHCl was found to be 29.6 cc per g, in agreement with the value of 29 cc per g predicted by the empirical relationship given by Tanford et al. (32). The agreement between the empirical molecular weight deduced from gel chromatography and the true molecular weight in 6 M GuHCl measured by sedimentation equilibrium shows that the Stokes radius is that expected for a random coil. Fig. 4 shows that the circular dichroism induced by aromatic side chain interactions in the native state has disappeared. Fig. 3 shows the typical optical rotatory dispersion curve of a randomly coiled protein, without structural features in the 220- to 240-nm region or in the aromatic absorption region.

The difference spectrum between native and denatured states, shown in Fig. 5, is qualitatively similar to that observed for the human carbonic anhydrases and many other proteins. The three minima in the 270- to 300-nm region reflect exposure of buried aromatic residues (33). The 291.4-nm trough is mainly probably due to the exposure of buried tryptophyl residues. At this wave length the difference absorbance for tyrosyl residues is negligibly small. The other two minima at 286.4 and 280 nm are probably due to exposure of buried tyrosyl as well as tryptophyl residues. The origin of the much larger trough at 235 nm has been the subject of some controversy. Glazer and Smith (34, 35) attributed it to the helix-coil transition of the polypeptide backbone, and this interpretation has been supported by Bailey et al. (36). Eisenberg and Edsall (37), however, suggested that the major contribution to the change in absorption in the 230- to 240-nm region has the same origin as the spectral difference in the 270- to 310-nm region and this interpretation is supported by the recent careful investigation of this problem by Donovan (14), who concluded that the absorption changes observed near 230 nm for globular proteins result primarily from changes in the environment of the aromatic chromophores indole and phenol and that the contribution from backbone helix-coil transition is less than 10% of the absorption changes typically observed. The data of this study (see below) support this latter interpretation.

Transition Equilibrium by Difference Spectroscopy—The transition was followed by difference spectral measurements at 291.4, 286.4, and 235 nm. Fig. 6 shows the results at 291.4 nm, in terms of the fraction (\( f_u \)) of the total change that has occurred at each particular GuHCl concentration at which measurements were made. Thermodynamic reversibility is established by the fact that solutions exposed to high GuHCl concentration and then diluted (filled circles) give \( f_u \) values which agree with those obtained at the same final GuHCl concentration without prior exposure to high denaturant concentration. The transition profile obtained at the other two wave lengths was essentially identical with that shown in Fig. 6. The results are shown in Fig. 7.
In view of the distinctly different profile obtained from optical rotation measurements that can clearly be identified with the conformational change of the polypeptide backbone, the similarity between the results at 235 nm and at the other two wave lengths is comprehensible only if the spectral change at 235 nm primarily reflects the altered environment of aromatic residues, in agreement with the conclusions of Donovan (14) cited above.

**Transition Equilibrium by Circular Dichroism**—The denaturation profile as monitored by circular dichroism measurements at 269 nm is shown in Fig. 6. Similar results were obtained when other wave lengths in the aromatic spectral region of Fig. 4 were employed. The transition is seen to take place at lower denaturant concentration than that measured by difference spectroscopy, with a midpoint at 1.4 M GuHCl as compared to the midpoint of 1.8 M GuHCl found by the latter method. It is evident that the asymmetrical interactions which lead to optical activity of the aromatic residues are relaxed at a lower concentration of denaturant than is required for exposure of these residues to the solvent.

**Transition Equilibrium by Optical Rotation**—The denaturation of carbonic anhydrase is accompanied by loss of the aromatic Cotton effects at 270 to 300 nm as well as by a major change at shorter wave length that reflects the randomization of the polypeptide backbone. As Fig. 3 shows, the magnitude of the change in \([m']\) that is due to disappearance of the aromatic Cotton effects is only a small fraction of the total change even in the aromatic absorption region, and changes in \([m']\) at all wave lengths thus reflect primarily the changes in folding of the polypeptide backbone. The denaturation profile shown in Fig. 6 was determined at 400 nm, but again similar results were obtained at several other wave lengths as shown in Fig. 8. The change in the structure of the polypeptide backbone is seen to occur at a denaturant concentration higher than required for exposure of aromatic residues. The filled triangles in the figure represent a test of reversibility and show that this difference represents a difference in the true equilibrium states at each concentration of GuHCl.

**Transition Equilibrium by Activity Measurement**—The loss of specific esterase activity during denaturation is shown in Fig. 9. The transition monitored in this way is seen to be separated into two steps. The second step is likely due to destruction of the conformation integrity of the active site but is difficult to correlate with any of the changes in CD, \(\Delta \varepsilon\), or ORD due to uncertainty arising from the large change in the first step. The hyperbolic shape of the first step change is probably due to inhibition of enzymatic activity by GuHCl, since this reagent has been alleged to be a potent inhibitor of carbonic anhydrases (38). The renatured protein was found to be indistinguishable from the native enzyme by circular dichroism, optical rotatory dispersion, and ultraviolet absorption. Recovery of specific esterase activity was better than 90%.

**Preliminary Kinetic Data**—Fig. 10 shows a semilogarithmic plot of the time course of refolding of randomly coiled enzyme at

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**Fig. 7.** Denaturation profiles of carbonic anhydrase B by GuHCl as followed by change spectral change at 291.4, 286.4, and 235 nm.

**Fig. 8** (left). Denaturation profiles of carbonic anhydrase B by GuHCl as followed by changes of optical rotation at 400, 350, 300, and 287 nm.

**Fig. 9** (center). Denaturation profile of carbonic anhydrase B by GuHCl as followed by percentage of activity loss. ■, solutions which have been exposed to high GuHCl concentration and diluted.

**Fig. 10** (right). Kinetics of refolding at 0.61 M GuHCl, pH 7.0, and 25°C. ○, the theoretical zero time value.
0.61 M GuHCl, as measured by ultraviolet absorbance at 291.4 nm. The filled circle represents the theoretical zero time value of the absorbance. It is evident that the reaction is kinetically very complex and that a minimum of three exponential decay terms (98) is required to describe the time course of the reaction. Similar results have been obtained in kinetic studies of the unfolding reaction.

**DISCUSSION**

The results presented in this paper have shown that the interconversion of native and randomly coiled bovine carbonic anhydrase B is a reversible process that can be separated into distinct successive stages both by equilibrium and by kinetic measurements. The equilibrium data suggest that a loosening of the tight folding of the native structure, leading to abolition of the circular dichroism arising from aromatic side chains, occurs as a separate process preceding the exposure of the aromatic side chains to the solvent. The disruption of the secondary structure (folding of the polypeptide backbone) represents a third stage of the reaction, at a distinctly higher concentration of GuHCl than the processes that involve the aromatic side chains. Moreover, it has been confirmed that carbonic anhydrase contains neither disulfide bonds nor free —SH groups, nor is there any evidence for formation of polymeric species. The unfolding and refolding of this protein therefore occur without complications from disulfide bond formation or from side reactions involving free —SH groups. The over-all reaction must consist of successive unimolecular steps, each representing a pure conformational change. It is evident that carbonic anhydrase is uniquely suited for investigation of the mechanism whereby an unfolded polypeptide chain adopts its native conformation. The high degree of cooperativity that prevents direct observation of this process for most globular proteins is evidently not present in carbonic anhydrase.

A possibly complicating factor is the presence of an atom of zinc in the native enzyme, and one of the stages of the reaction, both thermodynamically and kinetically, could involve the reversible association of zinc with the protein. This factor has not been investigated in these studies, but it should present no experimental difficulty in that the role of zinc in the transition can readily be assessed by varying the zinc content of the medium.

As part of this work the properties of the native enzyme were re-examined. A molecular weight of 29,000 was obtained, slightly smaller than the value of 31,000 obtained by Lindskog (40) by the approach to equilibrium method or the values reported by Reynaud et al. (41). Part of this difference is due to our use of a smaller value for $\varepsilon$. If we had used Lindskog’s pycnometrically determined value of 0.742 cc/g, a molecular weight of 30,000 would have been obtained. The value of $d_{40}^0$ obtained by us is 2.89 S, also lower than Lindskog’s value of 3.07 S (40), and Reynaud and co-workers’ value of 3.2 S (41).

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