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Renaturation of Bovine Erythrocyte Carbonic Anhydrase B Denatured by Acid, Heat, and Detergent†

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ABSTRACT: The renaturations of bovine carbonic anhydrase B denatured by acid, heat, and detergent have been studied in a continuing effort to elucidate the folding mechanism of this protein. Complete loss of enzymatic activity was observed when the native protein was acidified to pH 2, heated to 70 °C, or treated with 0.025% and 0.1% sodium dodecyl sulfate. These denatured proteins possess conformation states which are different from the native conformation and the random-coiled state of the protein. They have been obtained both from the native protein directly and from the random-coiled protein and shown to be the same regardless of the initial protein conformation. The results suggest that the native conformation is not required for the formation of the unique conformations of the partially denatured proteins. The renaturation of the denatured proteins by direct reversal to native conditions generally yields an enzymatically inactive protein with conformations different from the native one, and sometimes results in aggregation and precipitation. The precipitates formed upon renaturation by reversing the pH to neutral or cooling the protein back to room temperature may be states first attained kinetically upon renaturation. The dissolution of the precipitate is then kinetically too slow to enable complete renaturation. However, these denatured proteins can be completely renatured to the native conformation with total recovery of enzymatic activity if they are first converted to the random-coiled state by guanidinium chloride. The results raise the possibility of "irreversible" denaturation of proteins that are exposed to these denaturants during isolation and purification and suggest a general method for renaturing the otherwise "irreversibly" denatured proteins. As part of this work, the high and low detergent-binding states of carbonic anhydrase have been characterized by UV difference spectroscopy, by near- and far-UV circular dichroism spectra, and by intrinsic viscosity. The thermal denaturation profile of this protein was established by the $\Delta pH/\Delta T$ method, and the melting temperature (T_m) was determined to be 64.3 °C.

The last step in protein synthesis is the folding of the newly synthesized polypeptide chain into a unique conformation. The demonstration of the existence of intermediate conformations in the in vitro folding of a random-coiled protein will enable a detailed study of how the genetic information encoded in the amino acid sequence is expressed in the formation of the unique native conformation [e.g., Baldwin (1975) and Wong & Tanford (1970, 1973)]. Such intermediate conformations show which parts of a refolding protein are formed first and indicate what types of interactions stabilize these intermediates. Moreover, they may provide information on the nucleation sites for the folding of other parts of the protein [e.g., Wetlaufer & Ristow (1973)].

Studies on the reversible denaturation of bovine erythrocyte carbonic anhydrase (Wong & Tanford, 1970, 1973; McCoy & Wong, 1979) have shown that the protein can be denatured to the random-coiled state by 6 M guanidinium chloride (GdmCl).[1] Furthermore, they showed that this unfolding–refolding equilibrium transition consists of separable, intermediate steps. Preliminary kinetic investigations of denaturation and renaturation of carbonic anhydrase (Wong & Tanford, 1970, 1973; Yazgan & Henkens, 1972; Wong et al., 1972a,b; Carlsson et al., 1973; Wong & Hamlin, 1975; Ko et al., 1977; Ikai et al., 1979; McCoy et al., 1980) have established the existence of kinetic intermediate states, including an "incorrectly folded" conformation which appears to exist in the transition region.

The acid denaturation of carbonic anhydrase leads to extensive aggregation (Riddiford, 1964; Coleman, 1965; Edsall et al., 1966; Wong & Hamlin, 1974). Subsequent to acid denaturation, efforts to renature the protein by direct reversal

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[1] Abbreviations used: GdmCl, guanidinium chloride; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism.

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to neutral pH lead to precipitation (Nilsson & Lindskog, 1967; Wong & Hamlin, 1974). The protein was also reported to be irreversibly denatured under some circumstances. Heating the protein to 65 °C, for example, causes it to precipitate (Davis, 1963), and the precipitate remains if the protein is subsequently cooled. Precipitation has also been observed by Ikai et al. (1979) if the protein is renatured from the random-coiled state in 6 M GdmCl to intermediate concentrations of GdmCl and subsequently renatured to the native medium. Although the denaturation of many proteins by detergent has been studied [e.g., Steinhardt & Reynolds (1969) and Lapanje (1978)], similar investigations on carbonic anhydrase have not been reported.

The present paper reports systematic studies of the denatured states of bovine carbonic anhydrase B induced by acid, heat, and detergent. Particular emphasis was placed on (1) characterization of the denatured states obtained from the native proteins, and those obtained from the random-coiled protein, and (2) the renaturation of the denatured proteins by two different pathways: (a) by direct reversal to native conditions and (b) by first converting them to the random-coiled state by 6 M GdmCl followed by subsequent removal of the GdmCl.

Experimental Procedures

Materials. Bovine carbonic anhydrase B was purified from fresh bovine blood obtained from a single cow following the procedures described earlier (Henderson & Henriksson, 1973; Wong & Hamlin, 1974). The purified protein was shown to be homogeneous as determined by disc polyacrylamide gel electrophoresis and sedimentation velocity analyses. The protein was stored in a suspension of saturated $(NH_4)_2SO_4$ solution at 3–5 °C.

Ultrapure GdmCl was obtained either from Heico or from Schwarz/Mann, satisfying the criteria of purity (Wong et al., 1972a,b). $NaDodSO_4$ was obtained from Pierce Chemical Co. as a sequanal grade. All other chemicals were analytical or reagent grade and used without further purification. Water was glass distilled and passed through a Continental deionizer and organic remover before redistilling.

Methods. Specific esterase activity was assayed according to the procedure of Pocker & Stone (1965) with modifications (Wong & Hamlin, 1974).

CD[1] measurements were made on a Jasco Model J-20 CD spectropolarimeter. The instrument was calibrated by using a 1 or 0.1 mg/mL solution of *d*-10-camphorsulfonic acid (Cassim & Yang, 1969). All cells were cylindrical and strain free, with a path length ranging from 0.1 to 1 mm for measurements in the far-UV region and 1 cm for the near-UV region. The absorbancies of all solutions were kept below 1.5. The cell compartment temperature remained constant at 26 ± 1 °C. Observed ellipticity values were converted to mean residue ellipticity, $[\theta]_{MRW}$, based upon a molecular weight of 29 100 and 259 amino acid residues per molecule of bovine carbonic anhydrase B (Sciaky et al., 1976).

Ultraviolet difference absorption spectra were obtained according to the procedures of Herskovits & Laskowski (1962) by using tandem cells or matched pairs of 1-cm rectangular quartz cells with a Cary 14 double-beam spectrophotometer at 25 ± 1 °C. The total absorbance was kept below 2. Difference spectra were ascertained by exchange of the sample and reference cells from the corresponding cell compartments.

Viscosity measurements were made by using Cannon-Manning semimicro viscometers equilibrated with a Tamson Model TEV 70 or Cannon Model M-1 constant-temperature bath thermostated at 25 ± 0.005 °C. Flow times ranged from 266 to 486 s with five to eight determinations made at each protein concentration. Intrinsic viscosity, $[\eta]$, was related to the hydrodynamic volume of the protein by eq 1 (Tanford, 1961) where ν is the Simha shape factor, $\bar{v}_2$ is the partial specific volume of the protein, δ_2 is the grams of water per gram of protein, $v_1°$ is the specific volume of water, δ_2 is the grams of $NaDodSO_4$ per gram of protein, and $v_2°$ is the specific volume of $NaDodSO_4$. Appropriate values were taken from Reynolds & Tanford (1970a). The particle volume can then be calculated by eq 2 where M_r is the molecular weight of the protein and N is Avogadro's number. The symbols a and b refer to the major and minor axes, respectively. Treated as a prolate ellipsoid, eq 3 (Tanford, 1961) can be applied,

$$[\eta] = \nu(\bar{v}_2 + \delta_1 v_1° + \delta_2 v_2°) \quad (1)$$

$$M_r/[N(\bar{v}_2 + \delta_1 v_1° + \delta_2 v_2°)] = \tfrac{3}{4}\pi ab^2 \quad (2)$$

$$\nu = (J^2/15)(\ln 2J - \tfrac{3}{2}) + (J^2/5)(\ln 2J - \tfrac{1}{2}) + \tfrac{14}{15} \quad (3)$$

where J in this equation refers to the axial ratio a/b. Solving these equations simultaneously gives the radii of the molecule.

Thermal denaturation profiles were obtained by the method of Bull & Breese (1973). The protein solution, 1–2 mg/mL in 0.1 N NaCl, was heated at a constant rate of 0.5 °C/min (±10%) by using a Lauda K-2/R water bath. The pH was determined by using a Radiometer PHM-25 pH meter standardized at pH 7.00 and 4.01. A Radiometer G-202CH high-temperature electrode and B-531 salt bridge were placed in the protein solution, and a K-4018 calomel electrode was used as a reference. Temperature was monitored by using a YSI 42-SC telethermometer.

Renaturation of the partially denatured proteins was also carried out by first converting them to the random-coiled protein by 6 M GdmCl addition, and then they were renatured by removal of the denaturant. These experiments were carried out by adding stock solutions of GdmCl to the partially denatured protein samples to render the solution in 6 M GdmCl. The pH of the solution was then adjusted to about 6.5 and the GdmCl removed by dialysis of the sample against 0.1 N NaCl at the same pH.

Routine pH measurements were made on a Radiometer PHM-26 pH meter with a GK2302CK combined glass electrode and calibrated by using standard pH 7.00 and 4.01 buffers. Computer analysis for the amounts of α helix and β structure was determined by a nonlinear least-squares analysis by employing the program reported by Chen et al. (1974). Protein concentrations were routinely determined by absorbance measurements by using $E_{280}^{1\%} = 18.3$ for the native protein (Wong & Tanford, 1973).

Results

Characterization of the Acid-, Detergent-, and Heat-Denatured States. The acid-denatured state has been defined previously as the conformation of the protein at pH 2 in 0.1 N NaCl (Wong & Hamlin, 1974). In the present study, two detergent-denatured states have been studied: the high-binding state is the state at 0.1% $NaDodSO_4$, and the low-binding state is at 0.025% $NaDodSO_4$. These various denatured states have been obtained from two completely different initial conformations, i.e., the native and the random-coiled conformations. A comparison of the denatured states obtained from these two different initial conformations should provide some understanding of the role of the native conformation in the formation of the denatured protein.

Enzymatic Activities. As shown in Table I, the denaturation of the protein by acid, temperature, and $NaDodSO_4$ results in a complete loss of enzymatic activity, regardless of whether

Table I: Enzymatic Activity of Various Denatured States of Bovine Carbonic Anhydrase B

samples	enzymatic activity (%)
native protein (0.1 N NaCl, pH 7.0)	100
random-coiled protein (6 M GdmCl)	0
acid-, heat-, and detergent-denatured states obtained directly from native proteins	0
acid-, heat-, and detergent-denatured states obtained via the random-coiled state	0

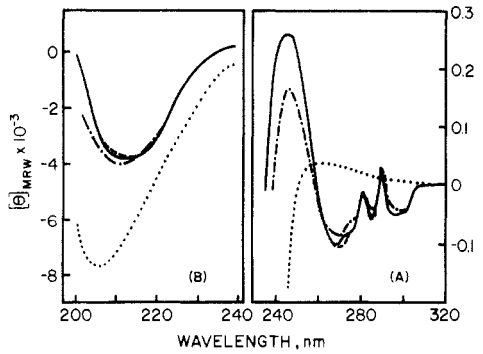

FIGURE 1: CD spectra of acid-denatured and renatured bovine carbonic anhydrase B: (A) near-UV CD; (B) far-UV CD. Native protein (—); acid-denatured protein obtained from the native protein by direct adjustment of the pH to 2 (···); renatured protein obtained via the random-coiled protein (- - -); renatured protein obtained by direct reversal to pH 7.0– (-·-·). Conditions: 0.1 N NaCl; protein concentration, 0.45–0.5 mg/mL; 25 ± 1 °C. Note that the dashed curve is completely identical with the solid curve except near 275 nm in (A) and near 210 nm in (B).

these partially denatured proteins are obtained from the native protein or from the random-coiled protein.

Conformation of Acid-Denatured Carbonic Anhydrase. The characteristic near-UV CD bands of the native protein (solid curve, Figure 1A) probably originate from the aromatic amino acid clusters in the tertiary structure of the protein (Beychok, 1966; Rosenberg, 1966; Lindskog et al., 1972). Upon acid denaturation, these complex ellipticity bands vanish and are replaced by a very broad and slightly positive curve (dotted curve, Figure 1A). The same featureless near-UV CD curve is also obtained for acid-denatured carbonic anhydrase from the random-coiled protein.

The far-UV CD spectrum of the native protein is shown as the solid curve in Figure 1B. The large broad trough with a minimum at about 215 nm is different from the characteristic double trough of proteins having significant amounts of α helix [e.g., Sears & Beychok (1973)] but is characteristic of proteins having the β structure as the predominant conformational features. As shown by the dotted curve in Figure 1B, acid denaturation of the protein leads to a 2-fold increase in ellipticity and a blue shift to a new minimum at 206 nm. These changes reflect considerable differences in the conformations of the acid-denatured and native states. Computer analysis of the far-UV CD spectra by the method of Chen et al. (1974) was made and shown in Table II. While the disordered structure remains the same, the α-helical content increases and is accompanied by a corresponding decrease of the β structure.

The far-UV CD spectrum of the acid-denatured protein obtained from its random-coiled state was also determined and shown to be the same as that from the native protein. The fact that the two acid-denatured proteins possess the same secondary structure suggests that the formation of secondary structure in acid-denatured carbonic anhydrase is independent of the native conformation. However, the possibility that folding of the random-coiled protein to the acid-denatured conformation may pass through the native conformation cannot be excluded.

Table II: Secondary Structural Estimation of Various Denatured States of Bovine Carbonic Anhydrase B

samples	% of secondary structure		
	α helix	β structure	disordered
native protein (0.1 N NaCl, pH 7)	~5	~35	~60
denatured proteins			
acid denatured (0.1 N NaCl, pH 2)	~15	~24	~61
high-binding detergent denatured (0.1% $NaDodSO_4$)	~34	~12	~54
low-binding detergent denatured (0.025% $NaDodSO_4$)	~30	~15	~55
random-coiled protein (6 M GdmCl)	~4	~14	~82

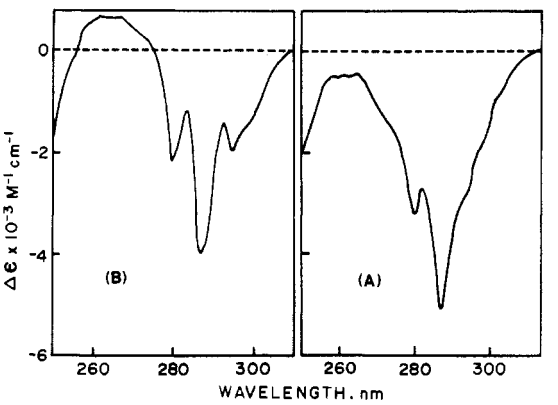

FIGURE 2: Different absorption spectra of $NaDodSO_4$-denatured bovine carbonic anhydrase B: (A) low-binding state; (B) high-binding state. Other conditions are the same as in Figure 1.

Hydrodynamic and Conformational Properties of High and Low Detergent-Binding Carbonic Anhydrase. Detergents are known to cause major conformational changes in proteins. The present report considers primarily two *distinct* conformations which exist in the presence of high and low concentrations of $NaDodSO_4$. The existence of multiple conformations as a function of the $NaDodSO_4$ concentration has previously been reported by Reynolds & Tanford (1970a) and by Takagi et al. (1975).

The difference absorption spectrum of the low-binding $NaDodSO_4$-denatured state is shown in Figure 2A. The large trough at 286–287 nm and the smaller trough at 280–281 nm result predominantly from the exposure of tryptophanyl and tyrosyl residues to the solvent environments. The characteristic 292–295-nm trough normally observed as a result of the exposure of buried tryptophanyl residues has been reduced to a shoulder under these conditions.

The difference absorption spectrum of the high-binding state is shown in Figure 2B. Relative to the acid-denatured state, the spectrum is red shifted by 1–2 nm, indicating a somewhat less polar environment. The small peak at 294 nm has a difference molar extinction coefficient of ~2000 M^{-1} cm^{-1} and is due predominantly to the exposure of tryptophanyl residues. Both tyrosyl and tryptophanyl residues contribute to the troughs at 287 and 281 nm which have difference molar extinction coefficients of ~4000 and ~2200 M^{-1} cm^{-1}, respectively.

The near-UV CD spectrum for the low-binding state is shown by the dashed curve in Figure 3A. Unlike the other denatured states, there is native-like character in this region

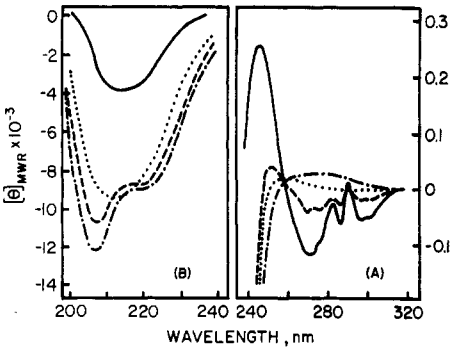

FIGURE 3: CD spectra of $NaDodSO_4$-denatured and renatured bovine carbonic anhydrase B: (A) near-UV CD; (B) far-UV CD. Native protein (—); low-binding $NaDodSO_4$-denatured protein (- - -); high-binding $NaDodSO_4$-denatured protein (····); renatured protein from the low-binding $NaDodSO_4$-denatured state by exhaustive dialysis (···). Other conditions are the same as in Figure 1. Note that the CD curves for the renatured protein obtained via the random-coiled state are the same as those of the native protein (solid curves).

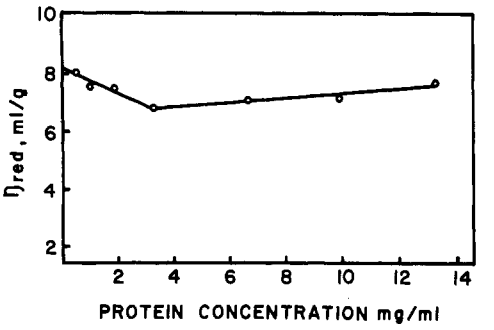

FIGURE 4: Intrinsic viscosity of bovine carbonic anhydrase B in 0.025% $NaDodSO_4$. Conditions are the same as in Figure 1.

of the CD spectrum. Above 260 nm, the CD curve is qualitatively the same as that of the native protein. The magnitudes of the ellipticity bands for this state are about one-third of those of the native protein. Below 260 nm, the CD spectrum begins to differ more from that of the native protein. The 245-nm peak present in the native enzyme is reduced drastically. The difference may be the result of overlapping with the large trough at the far-UV region.

The near-UV CD spectrum for the high-binding state is represented by the broken curve in Figure 3A and is similar to that of the acid-denatured state. The dashed curve in Figure 3B represents the far-UV CD spectrum for the low-binding $NaDodSO_4$-denatured protein. Ellipticity bands in this region do not resemble those of the native enzyme shown as the solid curve in the same figure. The spectrum does, however, closely resemble that of the high-binding state shown as the broken curve in the same figure. Computer analysis of the CD spectrum indicates 30% α helix and 15% β structure (Table II).

The far-UV CD spectrum of the protein in the high-binding state indicated the characteristic double trough, resembling typical proteins with large amounts of α helix (broken curve in Figure 3B). There is a comparatively large trough at 222 nm and another larger trough at about 208 nm. Reynolds & Tanford (1970b) have inferred from viscosity data that protein–$NaDodSO_4$ complexes may have very large amounts of α helix while Mattice et al. (1976) indicate that the amount of α helix as estimated by $[\theta]_{222}$ may be highly variable, depending upon the protein. Computer analysis (Table II) estimates that this conformation contains about 34% α helix and 12% β structure.

Figure 4 shows a plot of reduced viscosity, $\eta_{red.}$, vs. concentration, c, and extrapolated to zero concentrations to give an intrinsic viscosity of 8.1 cm^3/g for the low-binding state. There is a break in the curve at a protein concentration of ~3 mg/mL, producing a negative slope at lower protein concentrations. This break has been observed for other proteins in $NaDodSO_4$ (Reynolds & Tanford, 1970a). However, the explanation for this phenomenon remains unknown.

For interpretation of the intrinsic viscosity properly, the treatment of proteins in $NaDodSO_4$ as prolate ellipsoids was employed (Tanford, 1961). For the low-binding state, a value of 14 Å was obtained for the minor axis, and a value of 76 Å was obtained for the major axis. These values agree with those reported by Reynolds & Tanford (1970a) and indicate that bovine carbonic anhydrase B behaves hydrodynamically in a manner similar to those proteins used in their investigation.

The intrinsic viscosity of the protein in the high-binding state was also determined and found to have a value of 18.1 cm^3/g, which is intermediate between the native and the random-coiled states. Since micelles existed in the high-binding state, it was necessary to equilibrate the protein with the solvent. This was accomplished by passing the $NaDodSO_4$-denatured protein through a Sephadex G-10 column which had been equilibrated with the denaturing medium. The treatment of the data as a prolate ellipsoid gives a value of 17.5 Å for the minor axis and 103 Å for the major axis of the high-binding $NaDodSO_4$-denatured state. These values again are in agreement with the values for other proteins obtained by Reynolds & Tanford (1970a). It should be noted that our data in no way distinguish this prolate ellipsoid model from the others (Takagi et al., 1975; Wright et al., 1975; Mattice et al., 1976). One of these models which proposes that the $NaDodSO_4$–protein complex exists as a "necklace" or "free-draining flexible polypeptide" has received further support from electric birefringence studies (Rowe & Steinhardt, 1976).

The high-binding $NaDodSO_4$ state could be obtained by exhaustively dialyzing the random-coiled protein in 6 M urea in 0.1% $NaDodSO_4$ against an excess of 0.1% $NaDodSO_4$ in 0.1 N NaCl. The use of urea was necessitated by the precipitation of $NaDodSO_4$ in 6 M GdmCl. The CD spectra of the high-binding $NaDodSO_4$ state obtained from 6 M urea are indistinguishable from those of the high-binding $NaDodSO_4$-denatured state obtained directly from the native protein (see broken curves in Figure 3A,B).

Heat-Denatured Carbonic Anhydrase. The precipitation of carbonic anhydrase upon heating to 65 °C has been reported (Davis, 1963) and has been observed in our heat denaturation study. The precipitate remains upon further heating to 95 °C. This, unfortunately, makes spectroscopic studies impossible. However, heat denaturation can be determined by the differential pH method (Bull & Breese, 1973). Buried residues in the native state may become exposed to the solvent during denaturation and thereby remove or add hydrogen ions to the solvent. The thermal denaturation pH profile for the protein is shown by the upper panel in Figure 5. As represented by the shaded portion of the curve, the protein begins to precipitate from the solution at about 55 °C. The total change in pH is 0.38 unit with a minimum value of 6.49 and a maximum value of 6.87. The protein unfolds in the range of 55–70 °C with a midpoint, T_m, at 64.3 °C as indicated by the peak position of the first derivative of the melting profile shown in the lower panel of Figure 5. The heat-denatured state is defined as the state obtained by heating the protein to 70 °C.

In order to obtain the heat-denatured protein from its random-coiled state, bovine carbonic anhydrase B in 6 M GdmCl was dialyzed against a 100-fold excess of 0.1 N NaCl at 70 °C; it precipitates out of solution in a matter of a few

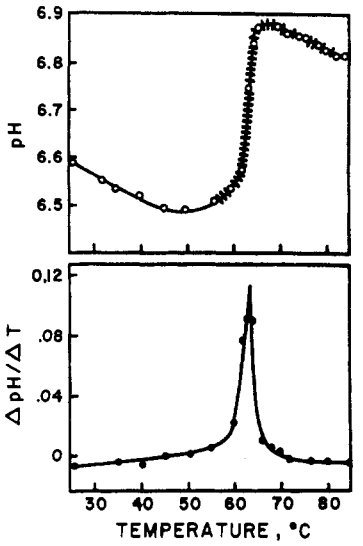

FIGURE 5: Thermal denaturation profile of bovine carbonic anhydrase B. Protein concentration, 7.1 mg/mL in 0.1 N NaCl. Shaded portion of the profile in the upper panel represents the temperature range where precipitation of the protein was observed.

Table III: Enzymatic Activity of Various Renatured States of Bovine Carbonic Anhydrase B

samples	enzymatic activity (%)
renatured directly	
from 0.1% to 0.025% $NaDodSO_4$ by dialysis	0
from 0.025% to 0.1 N NaCl, pH 7, by dialysis	0
from pH 2.0 to ~7 (at high protein concn)	0
from 70 °C by cooling (at high protein concn)	~2
renatured via 6 M GdmCl or 6 M urea	
from native enzyme	~99
from pH 2.0	~84–93
from 0.1% $NaDodSO_4$	~100
from heat-denatured protein	~100

hours. No studies were performed to characterize this heat-denatured state because of its insolubility.

Renaturation of Acid-, Heat-, and Detergent-Denatured Carbonic Anhydrase. Two methods for renaturation were used, i.e., (1) direct renaturation of these denatured proteins by returning to the native medium (0.1 N NaCl, pH 6–7, 25 ± 1 °C) and (2) indirect renaturation by first converting the denatured proteins to the random-coiled state and subsequently dialyzing the random-coiled protein against the native medium.

Enzymatic Activity. The specific esterase activity of renatured carbonic anhydrases which have been denatured by acid, heat, and detergent and either obtained directly or obtained via the random-coiled state has been assayed and summarized in Table III. The results show that direct renaturation yielded completely inactive proteins, while complete or almost complete recovery of activity was obtained if the denatured proteins were first converted to the random-coiled state.

Renaturation by Direct Reversal to Native Conditions. When the acid-denatured protein was directly renatured by adjusting the pH to neutrality, precipitation of the protein occurred above pH 4.2 as was observed in previous studies (Wong & Hamlin, 1974; Nilsson & Lindskog, 1967). Subsequent to filtration of the renatured protein, the enzymatic activity was determined, and little or no activity was found. The concentration of the soluble renatured protein was so diluted (<1 μg/mL) such that reliable spectroscopic information could not be obtained.

Table IV: Secondary Structural Estimation of Various Renatured States of Bovine Carbonic Anhydrase B

samples	% of secondary structure: α helix	β structure	disordered
native protein	~5	~35	~60
renatured proteins obtained directly			
from 0.1% $NaDodSO_4$ by dialysis	~25	~29	~46
from 0.1% to 0.025% $NaDodSO_4$	~30	~15	~55
renatured protein obtained via the random-coiled state			
from the heat-denatured state	~5	~35	~60
from the acid-denatured state	~5	~35	~60
from the high-binding $NaDodSO_4$-denatured state	~5	~35	~60

Dilution of the high-binding state in 0.1% $NaDodSO_4$ to 0.025% results in the formation of near- and far-UV CD spectra which are indistinguishable from the low-binding state obtained from the native enzyme (see dashed lines of Figure 3A,B). The complete renaturation of the detergent-denatured protein would require that the bound $NaDodSO_4$ be completely removed since even minute amounts of bound $NaDodSO_4$ could induce large conformational change (Tanford, 1968). If the binding were sufficiently weak, the $NaDodSO_4$ could be removed by dialysis. However, direct renaturation of the low-binding detergent state by dialysis does not lead to the recovery of the native conformation. Shown as the dotted curve in Figure 3A is the near-UV CD spectrum of the protein in the resulting denatured state. The ellipticity bands observed in the low-binding detergent-denatured state are not in evidence, suggesting that the tertiary structure of the protein has been disrupted. The prominent negative ellipticity band at 208 nm in the conformations at higher $NaDodSO_4$ concentrations is greatly diminished as can be seen by the dotted curve in Figure 3B. Computer analysis of this spectrum indicates ~25% α helix and ~29% β structure (Table IV).

The precipitate formed by heating the protein above 70 °C does not redissolve when the protein is slowly cooled back to room temperature. If the precipitate is removed by filtration, only minute amounts of protein remain in solution.

Renaturation via the Random-Coiled State. The renaturation of the acid-denatured protein by first converting it to the random-coiled state was studied by comparing the near-UV and far-UV CD spectra of the renatured protein with those of the native protein. The near- and far-UV CD spectra shown as the dashed curves in Figure 1A,B are the same as those of the native protein (solid curve) in the same figures.

Since $NaDodSO_4$ is insoluble in concentrated solutions of GdmCl, the solution of $NaDodSO_4$-denatured protein was made 6 M in urea, and the removal of $NaDodSO_4$ was carried out by passing the $NaDodSO_4$-bound protein through a Dowex AG-1-2 ion-exchange column by using 6 M urea as an eluant (Weber & Kuter, 1971). As seen in Table III, the enzymatic activity is completely restored upon renaturation.

When concentrated stock GdmCl solution is added to the precipitated heat-denatured protein to a GdmCl concentration of 6 M, the protein dissolved readily. Subsequent dialysis of the solution to remove the GdmCl results in complete recovery of the enzymatic activity (Table III). The near-UV CD spectrum and the far-UV CD spectrum show that within ex-

perimental error they are the same as the native spectra, indicating that the native conformation is quantitatively recovered. The UV absorption spectrum of the renatured protein is also identical with the spectrum of the native protein.

Discussion

The results show that the acid-, heat-, and detergent-denatured states of bovine erythrocyte carbonic anhydrase B assume conformations which are different from those of the native and random-coiled states. It is also demonstrated that the formation of these conformations resides solely on the amino acid sequence of the protein, and the conformations are formed according to the environmental conditions, regardless of the initial conformational state.

The aggregation resulting from the failure to renature the acid- and heat-denatured proteins by direct reversal to native conditions may be due to intermolecular interactions between one or more of the intermediate states. The strong binding of $NaDodSO_4$ molecules to proteins and the difficulties in removing the $NaDodSO_4$ molecule from proteins by simple dialysis or dilution have been well documented (Weber & Kuter, 1971). It is also possible that the kinetics of going to the trapped states are much faster than the kinetics of renaturation from those states.

The seemingly "irreversible" denaturation of bovine carbonic anhydrase B by acid, heat, and detergent as judged by the complete lack of enzymatic activity upon direct renaturation may have significant relevance to other proteins after their exposure to these denaturants during isolation, purification, and other manipulations. Attempts to renature the "irreversibly denatured" proteins via the random-coiled state should be followed as suggested by the successful renaturation of bovine erythrocyte carbonic anhydrase B from its "irreversible denatured" states.

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