# Acid Denaturation of Bovine Carbonic Anhydrase B†

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ABSTRACT: The acid denaturation of bovine carbonic anhydrase B has been studied by viscosity, difference spectrophotometry, circular dichroism, and enzymatic activity. Acid denatured bovine carbonic anhydrase B is enzymatically inactive. It does not exist in the random-coiled state as indicated by viscosity and circular dichroism. The course of denaturation was monitored from pH 8 to 2. A single conformational transition was observed at pH 4.8–4.0 based on circular dichroism changes in the aromatic region. However, difference spectrophotometry and circular dichroism studies of the peptide region revealed two separate stages of conformational transition. In addition to a transition similar to that revealed by aromatic circular dichroism studies (transition I), a second transition was observed at about pH 3.4–2.5 (transition II). It appears that upon addition of acid, the compact globular protein molecule undergoes an expansion (transition I) which results in the destruction of the asymmetric aromatic clusters in the molecule, the exposure of some buried aromatic residues to solvent, and the partial loosening of the conformation. On further addition of acid, the expanded molecule unfolds (transition II) to give the acid-denatured conformation which has a higher degree of exposure of buried aromatic residues and further loosening and unfolding of the molecule.

Bovine carbonic anhydrase B (carbonate hydrolyase, EC 4.2.1.1) is a zinc metalloenzyme which catalyzes the hydration of $CO_2$. It is a single chain protein with molecular weight of 29,000 and devoid of disulfide and sulfhydryl groups. The native enzyme is a compact globular protein as indicated by hydrodynamic measurements ($f/f_{min} = 1.14$, $[\eta] = 3.67$ cm$^3$/g) and possesses characteristic optical rotatory despersion (ORD) and circular dichroism (CD) spectra (Lindskog *et al.* 1971; Wong and Tanford, 1973). It has been shown to be a suitable protein for *in vitro* studies of how a functional protein which possesses a unique conformation is formed from its random-coiled polypeptide chain containing solely its amino acid sequence. The denaturation of bovine carbonic anhydrase B by Gdn·HCl[1] has been studied by Wong and Tanford (1970, 1973). They have shown that the process is completely reversible and that the denaturation product is a random-coiled polypeptide. Equilibrium studies of denaturation have unambiguously demonstrated the presence of stable intermediate states since significantly different transition profiles have been obtained when different parameters (CD, ORD, and difference spectra) were used to monitor the transition. The same conclusion was obtained from initial kinetic studies. The role of Zn(II) in the folding of bovine carbonic anhydrase B has been investigated (Yazgan and Henkens, 1972; Wong *et al.*, 1972; K.-P. Wong and L. M. Hamlin, manuscript submitted for publication). Comparative studies of the native enzyme, the apoenzyme, and the regenerated enzyme suggest no significant conformational differences. However, removal of zinc from the native enzyme decreases its conformational stability toward denaturation by Gdn·HCl. Initial kinetic study also indicates that the folding of the random-coiled polypeptide without zinc proceeds at a slower rate. The Zn(II) ion appears to increase the rate of folding and stabilizes the protein molecule but does not seem to be a necessary part of the information required for the folding process. The inactivation of crude bovine carbonic anhydrase by acid has been studied by activity measurements (Keller and Gottwald, 1960). Their results suggest that the reactivation of the acid-treated enzyme depends on the time, temperature, $H^+$ concentration, nature of the anion, and the concentration of protein. More recently, Nilsson and Lindskog (1967) studied the hydrogen ion titration of the pure enzyme and found that seven buried uncharged histidyl residues are protonated with a concomitant expansion of the molecule near pH 4.0 as indicated by a decrease of the electrostatic parameter, $\omega$. The existence of an intermediate conformation state of the enzyme at pH below 4 has been reported by ORD studies (Rosenberg, 1966). In this paper we present results on a detailed study of conformational changes as a function of pH by viscosity measurements, CD, ultraviolet difference spectroscopy, and activity assays. The acid-denatured state was characterized. The conformational transition was monitored from approximately pH 8 to 2, and from this a stable intermediate state was identified and characterized.

## Experimental Section

*Materials.* Bovine carbonic anhydrase B was purified from fresh bovine blood obtained from a single cow. The method for the preparation of human carbonic anhydrases (Armstrong *et al.*, 1966) was adopted. In later experiments the enzyme was prepared by the recent procedure of Henderson and Henriksson (1973) for the large-scale preparation of human carbonic anhydrases. All other chemicals used were either analytical or reagent grade. The water used was double distilled and deionized.

*Methods.* Viscosity measurements were made using Cannon-Manning semimicro capillary viscometers equilibrated in a Tamson Model TEV 70 constant-temperature bath thermostated at 25.00 ± 0.005°. Solvent flow time of the viscometers ranged from 220 to 380 sec. Flow time for protein solutions over that of solvent ranged from 11 to 18 sec with a precision of ±0.05 sec. Generally, 4–8 flow time measurements were made on each solution. Ultraviolet difference spectra were obtained at 25 ± 1° with a Cary 14 double-beam spectrophotometer. The absence of stray light contribution was

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

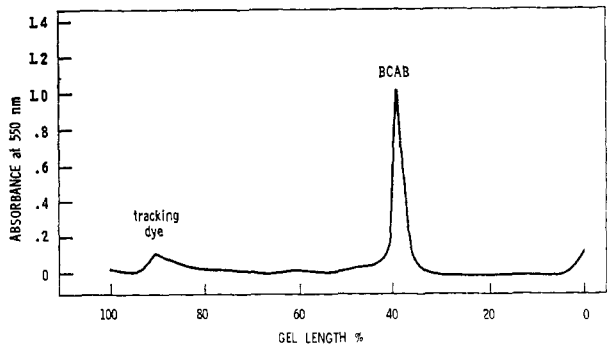


FIGURE 1: Spectrophotometric scan of a polyacrylamide gel of bovine carbonic anhydrase B. The single peak at about 40% gel length is the enzyme.

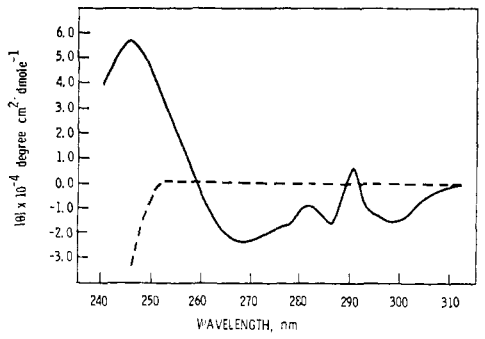


FIGURE 2: Near-ultraviolet CD spectra of bovine carbonic anhydrase B in 0.1 N NaCl: (—) the native enzyme, pH 7.0; (- - -) the acid-denatured enzyme at pH 2.0.

demonstrated by assuring that Beer's law was obeyed. The absorbancy was less than 2 at all wavelengths reported. Circular dichroism spectra were obtained from a JASCO Model J-20 CD spectropolarimeter at 26 ± 1°. Absorbance of solutions was less than 1.5. We expressed the results in terms of molar ellipticity, $[\theta]$, in units of deg cm²/dmol using the equation: $[\theta] = (M/dC)\theta$, where $\theta$ is the observed ellipticity in degrees, $M$ and $C$ are the molecular weight in g and concentration in g/ml, respectively, and $d$ is the optical path in decimeters. The CD spectropolarimeter was calibrated with a 1 mg/ml aqueous solution of *d*-10-camphorsulfonic acid to give a $[\theta]$ value of +7260 at 290.5 nm (Cassim and Yang, 1969). Denaturation studies were performed on a series of solutions with identical protein concentration but different pH values. These solutions were prepared by pipetting 5-ml portions of native protein solution from a common stock to separate small flasks, and then adjusting the pH individually from about 8 to 2 with HCl in small pH increments. The solutions were allowed to equilibrate at room temperature for about 12 hr to attain equilibrium. The pH values of each solution were measured after equilibration and no significant change of pH was obtained. All pH measurements were made with a Radiometer Model 26 pH meter and a general purpose combined glass electrode (GK2320C). The pH meter was standardized against pH 4.01 and 7.00 standard buffers. Protein concentrations were determined spectrophotometrically using an extinction coefficient of $A_{280}^{1\%} = 18.3$ (Wong and Tanford, 1973). Specific esterase activity of the enzyme (Pocker and Stone, 1965) was routinely assayed by monitoring the change in absorbance at 349 nm using *p*-nitrophenyl acetate as a substrate and acetazolamide as the specific inhibitor. For disc gel electrophoresis at pH 8.9, the method of Davis (1964) and Ornstein (1964) was employed.

## Results

*Large-Scale Preparation of Bovine Carbonic Anhydrase B.* The adoption of the method for the large-scale preparation of human carbonic anhydrase (Henderson and Henriksson, 1973) to the preparation of the bovine enzyme has yielded a satisfactory result. Normally 1.0–1.2 g of pure enzyme is obtained from 10 l. of fresh bovine blood. The purified protein was shown to be homogeneous with respect to disc gel electrophoresis and sedimentation velocity experiments. A typical spectrophotometric scan of the stained polyacrylamide gel is shown is Figure 1. There is no observable difference on the various physicochemical studies of the two enzyme preparations. The enzyme samples were stored in suspension of saturated $(NH_4)_2SO_4$ solution in the refrigerator (2–3°). Although we have not observed any difference in conformation and conformational stability between enzyme samples stored in $(NH_4)_2SO_4$ and those stored in lyophilized powder, we have occasionally detected change in solubility of the lyophilized enzyme. The lyophilized sample becomes less soluble under some conditions.

*Characterization of the Acid-Denatured State.* The conformation of the enzyme which exists at about pH 2.0–2.5 and in 0.1 N NaCl is defined as the acid-denatured state. No enzymatic activity was observed at this state.

The results of viscosity measurements were treated by plotting reduced viscosity, $\eta_{sp}/c$, *vs.* protein concentration, $c$. The reduced viscosity term is approximated by $\eta_{sp}/c = (t - t_0)/t_0c + (1 - \bar{v}\rho_0)/\rho_0$, where $t$ and $t_0$ are the flow time of solution and solvent, respectively, $\bar{v}$ is the partial specific volume of the solution, and $\rho_0$ is the density of solvent (Tanford, 1955). The value of $\bar{v}$ for the enzyme was calculated from its amino acid composition to be 0.733 cm³/g. The intrinsic viscosity of the acid-denatured enzyme obtained by extrapolation of the straight line to zero protein concentration is $[\eta] = 8.4$ cm³/g. The Huggin's constant, $k$, was calculated from the slope of the straight line to be 13.4.

The acid denaturation difference spectrum of the enzyme measured with the native enzyme in the reference cell is shown in Figure 6 by the dotted curve. In the 310–250-nm spectral region, three troughs with minima at 290, 286, and 280 nm are observed. The 290-nm trough has a molar absorbance change of about $-6500$ M$^{-1}$ cm$^{-1}$ and is probably due mainly to the exposure of buried tryptophyl residues. The 286- and 280-nm troughs have molar absorbance changes of about $-7400$ and $-5600$ M$^{-1}$ cm$^{-1}$, respectively, and both tryptophyl and tyrosyl residues are probably responsible for these troughs.

The CD spectra of the enzyme in the aromatic region are shown in Figure 2. The solid curve is the CD spectrum of the native enzyme. It has several ellipticity bands which presumably have the same origin as the multiple aromatic Cotton effects observed in ORD (Beychok *et al.*, 1966; Rosenberg, 1966). The dotted curve is the CD spectrum of the acid-denatured enzyme at pH 2.0. One notices that all the aromatic CD bands have virtually vanished indicating that the clustering of the aromatic amino acids is no longer in existence at this pH. Although the spectrum shows almost zero ellipticity value from 310 to 250 nm, we invariably observed small positive ellipticity values through all wavelengths. The origin of the positive CD band at about 245 nm is not known. This positive ellipticity band vanishes upon acid denaturation and actually acquires a negative ellipticity value, presumably due to the tailing of the large negative trough in the 240–200-nm region. The CD spectrum of the native enzyme in the 250–200-nm

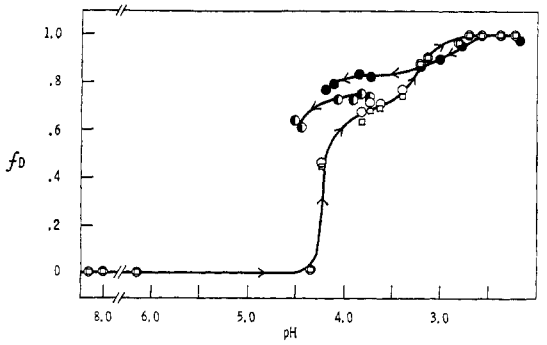


FIGURE 3: Denaturation of bovine carbonic anhydrase B as a function of pH monitored by difference absorption change at 290 nm (○) and at 287 nm (□): (●) the renaturation of the enzyme from pH 2; (○) the renaturation of the enzyme from pH 3.7; both were monitored by difference absorption change at 290 nm.

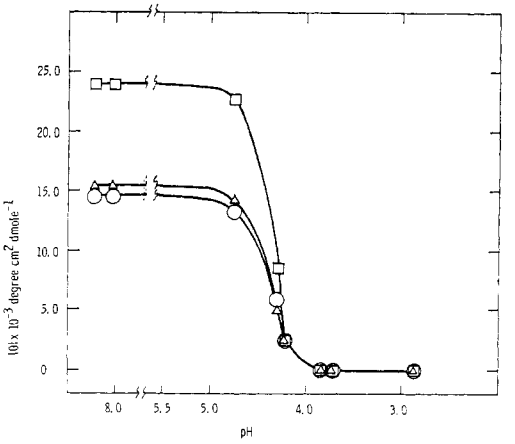


FIGURE 4: Change of aromatic CD of bovine carbonic anhydrase B as a function of pH monitored at 297 nm (□), 286 nm (△), and 270 nm (○).

region is shown by the solid curve in Figure 7. It does not bear any resemblance to the CD curves of typical globular proteins possessing a significant amount of $\alpha$-helical structure. The curve is shown to have a trough centered at 215–217 nm. Estimation of $\alpha$-helical content by the magnitude of ellipticity at 222 nm (Chen and Yang, 1971) results in a value of only 2–3%. In the acid-denatured enzyme this trough becomes more negative and simultaneously shifted to lower wavelength as shown by the dotted curve in Figure 7. The helical content at pH 2.0 is estimated by the same method to be approximately 7%. It should be noted that the above estimations of helical content were obtained by a procedure which does not take into account the possible contribution of aromatic amino acid residues to the CD spectra in this spectral region.

*Conformational Transition as a Function of pH.* The unfolding of the native enzyme to the acid-denatured state has been monitored by (i) difference absorbance changes at 290 and 287 nm which mainly reflect the exposure of buried aromatic residues to the aqueous solvent environment, (ii) changes in the ellipticity bands at 297, 286, and 270 nm which give indication on the integrity of the clusters of aromatic residues, and (iii) CD changes at 222 nm which presumably originate from the secondary and tertiary structure of the polypeptide backbone of the enzyme.

The transition profiles were monitored from pH 8.2 to 2.2 by the difference absorbance changes at 290 and 286 nm and are shown in Figure 3. The fraction of acid denaturation, $f_D$, is calculated by the equation, $f_D = (\Delta\epsilon_N - \Delta\epsilon_i)/(\Delta\epsilon_N - \Delta\epsilon_D)$, where $\Delta\epsilon_N$ and $\Delta\epsilon_D$ represent the difference absorbance changes of the native and the acid-denatured state, respectively; and $\Delta\epsilon_i$ is the difference absorbance change of the enzyme at any intermediate pH. As the pH of the solution was lowered, we occasionally observed precipitation at approximately pH 5.5 which redissolved below about pH 5.0. The acid denaturation of the enzyme is shown to occur in two distinct separable stages of conformational transition. The first transition, which accounts for approximately 70–75% of the total difference absorbance change, starts at pH 4.3, and is completed at about pH 3.8 with a midpoint at pH 4.1. The second transition occurs between pH 3.5 and 2.8 with a midpoint at about pH 3 and accounts for the remaining 25–30% of the total difference absorbance change. The transition profiles monitored at 290 and at 287 nm are identical.

The reversibility of these transitions was studied by back-titration of the enzyme from pH 2 to pH 3.7. During the adjustment of pH to neutral by addition of NaOH, the enzyme precipitated out of solution. However, the precipitate can be redissolved by raising the pH to 8 or above. It remains in solution when the pH is returned to 7.0. The specific esterase activity of the enzyme at pH 3.7 and 2.0 was assayed first and shown to be completely vanished. Upon renaturation of the enzyme to pH 7.0 after being kept at pH 2.2 for 30 min, only 26% of the activity was recovered. Recovery of activity after the enzyme was kept at pH 3.7 for 30 min was 39%. This partial recovery of activity is probably related to the incomplete renaturation monitored by difference absorbance measurements at 290 nm as shown in Figure 3. The renaturation profile from pH 2.2 is shown by filled circles. It is significantly different from the denaturation profile, being broader than the denaturation curve and regaining only 20–25% of the total change from pH 2.2 to 4.2. The protein precipitated out of solution when the pH was raised above 4.2 and remained insoluble to pH 8. Such irreversible behavior was also observed when the protein was renatured from pH 3.7 as shown by the half-filled circle in Figure 3. Again precipitation occurred above pH 4.5.

The acid denaturation was also followed by changes in CD spectra. Figure 4 shows the change of aromatic CD bands at 297, 286, and 270 nm as a function of pH. No difference is observed between the denaturation profile monitored at the three different wavelengths upon normalization of the curves. The magnitude of these troughs starts to decrease at pH 4.8 and completely disappears at about pH 4.0 with a midpoint at about 4.35. There is no further change observed at lower pH values. The change of CD in the peptide region was followed at 222 nm which reflects mainly the change of $\alpha$-helical structure of the enzyme, and the results are shown in Figure 5. The molar ellipticity begins a sharp change to more negative at about pH 4.3 and completes at about pH 4.0 with a midpoint at about pH 4.1. When the pH is further lowered another transition occurs between pH 3.2 and 2.5, with a midpoint at about pH 2.9. No significant change is observed below pH 2.5. It should be noted that in one or two occasions precipitation was observed around the isoelectric point of the protein which has been reported to be 5.65 (Nilsson and Lindskog, 1967) and 5.89 (Jonsson and Pettersson, 1968). However, the conformational transition region is completely outside this isoelectric precipitation of the protein.

*Characterization of the Intermediate State.* It is suggested from the conformational transition studies that there exists a stable intermediate conformational state at about pH 3.6 ± 0.2, which is enzymatically inactive and cannot be completely renatured. The difference absorption spectrum of the enzyme measured at this pH against the native enzyme is shown in

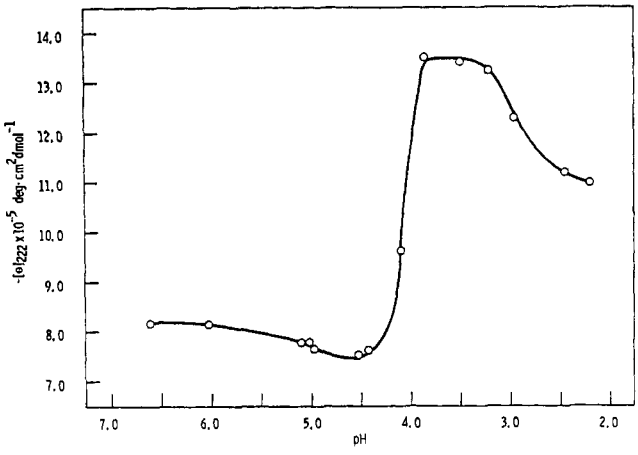


FIGURE 5: Molar ellipticity change of bovine carbonic anhydrase B at 222 nm as a function of pH.

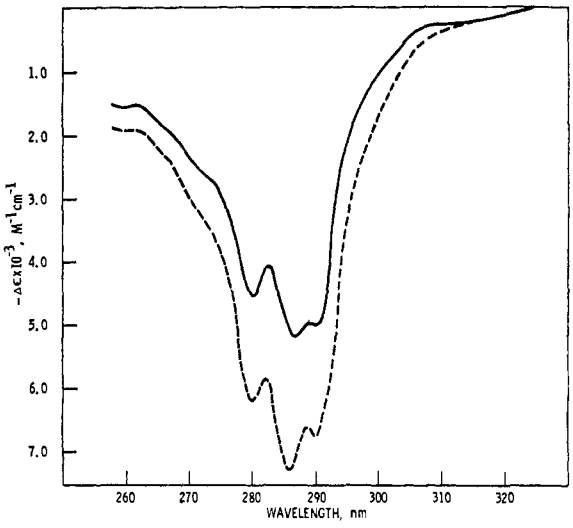


FIGURE 6: Near-ultraviolet difference spectrum of bovine carbonic anhydrase B at pH 3.7 (—), and at pH 2.23 (- - - -).

Figure 6 by the solid curve. It indicates intermediate properties between the native and the acid-denatured state. The CD spectrum of the enzyme at pH 3.7 in the aromatic region is shown to be the same as that in the acid-denatured state in which all the ellipticity bands are completely disappeared. The CD spectrum at the peptide region is shown in Figure 7 by the broken curve. It is similar in shape to the CD spectrum of the native enzyme with the magnitude of the ellipticity at 222 nm approximately two times larger. The viscosity of the enzyme at pH 3.6 ± 0.1 was also determined. Extrapolation of the linear plot of reduced viscosity *vs.* protein concentration to zero protein concentration yields an intrinsic viscosity value of $[\eta] = 4.14$ cm$^3$/g with a Huggin's constant, $k = 2.44$.

## Discussion

*Acid-Denatured State of Carbonic Anhydrase B.* The effect of acid on the conformational stability of different proteins may vary from retaining their native conformation to converting them to the drastically unfolded structural forms (Tanford, 1968). The results presented here suggest that acid-denatured bovine carbonic anhydrase B possesses a conformational state which is quite different from the native state and the random-coiled state as observed in 6 M Gdn·HCl. The intrinsic viscosity of 8.4 cm$^3$/g clearly indicates that the acid-denatured enzyme is not a random-coiled protein, since at such a state one would expect a value of about 29 cm$^3$/g or higher because of electrostatic repulsion at low pH (Tanford, 1968). Compared to earlier viscosity results of the native enzyme, $[\eta] = 2.7$ cm$^3$/g (Reynaud *et al.*, 1970) and $[\eta] = 3.7$ cm$^3$/g (Wong and Tanford, 1973), the acid-denatured enzyme has not retained its native structure. Such an intrinsic viscosity value is also outside the range found for most known globular proteins which normally fall within the range of 3.3–4.0 cm$^3$/g (Tanford, 1961). The Huggin's constant for the acid-denatured protein is also unusual, having a value of 13.4, and is significantly larger than the corresponding values for the native and the random-coiled enzyme which are 1.4 and 1.8, respectively. The acid-denatured enzyme shows typical blue-shifted ultraviolet absorption spectrum which indicates structural changes from the native enzyme involving the exposure of buried aromatic residues to the aqueous solvent environment. The difference absorption spectrum possesses some basic similarities to that of the random-coiled protein. However, the acid difference spectrum shows no positive absorption change at the 320–300-nm region which has been observed in the Gdn·HCl denaturation difference spectrum (Wong and Tanford, 1973). The acid denaturation difference spectra at the 270–250-nm region is also different. The acid-denatured enzyme possesses some negative fine structure while the random-coiled protein has positive values at this region. The magnitude of absorbance change at 290 and 286 nm is slightly below the corresponding value for the random-coiled protein at 6 M Gdn·HCl after the difference absorption spectrum of the Gdn·HCl denatured enzyme was corrected for the contribution of solvent effect. Recently, Lindskog and Nilsson (1973) studied the solvent perturbation difference spectra of carbonic anhydrase B and reported that the acid-denatured enzyme still retained a fraction of the aromatic residues shielded from the solvent. The CD of the acid-denatured enzyme suggests that it exists in a conformation significantly different from the native enzyme. The characteristic ellipticity bands in the aromatic region have completely vanished and the negative trough in the peptide region has

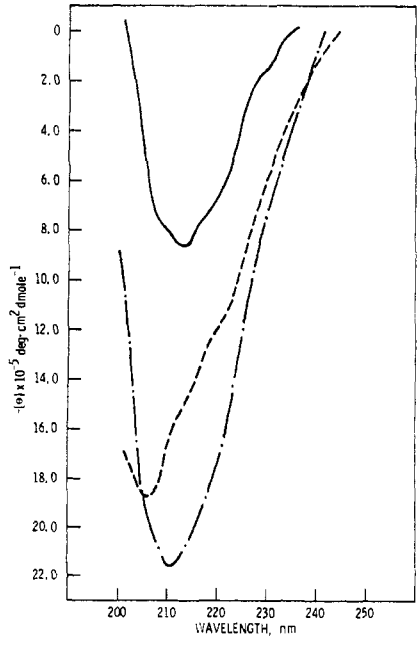


FIGURE 7: Far-ultraviolet CD spectrum of bovine carbonic anhydrase B at pH 3.7 (— · —), pH 2.0 (- - -), and pH 7 (—).

undergone a blue shift with concomitant increase in magnitude. However, the CD spectrum of the acid-denatured enzyme bears no resemblance to that of typical random-coiled proteins. The integrity of the active site conformation probably is destroyed in the acid-denatured state, since no specific esterase activity of carbonic anhydrase was observed. We have been unable to detect any precipitation of bovine carbonic anhydrase B at the acid-denatured state as indicated by the lack of light scattering and by ultraviolet absorption measurement from 350 to 310 nm. In light of reports on precipitation and aggregation of human carbonic anhydrase C and B upon acid denaturation (Riddiford *et al.*, 1965; Coleman, 1965), we are investigating the possibility of association of the bovine enzyme at low pH by sedimentation equilibrium measurements.

*The Conformational Transition as a Function of pH.* The major finding in the present study is that bovine carbonic anhydrase B undergoes two separate distinct stages of conformational transition upon acid denaturation. The first transition which occurs between pH 4.8–3.8 is reflected by the loss of enzymatic activity, a complete disappearance of the aromatic ellipticity bands, slight increase in intrinsic viscosity, the exposure of a significant number of buried aromatic residues to solvent, and an increase in the magnitude of the negative CD trough in the far-ultraviolet region without any shift in wavelength. The molecular expansion of the enzyme around pH 4 which involves the protonation of about seven buried uncharged histidyl residues as suggested by the potentiometric titration of Nilsson and Lindskog (1967) is probably another manifestation of this conformational transition. Although the exact molecular events which occur in this structural transition are difficult to decipher, some structural features can be inferred. One of the major driving forces is likely to be the protonation of the uncharged buried histidyl residues, since the stability of the expanded protein with protonated histidyl residues is favored. The integrity of the active site presumably is destroyed as a result of this transition since no enzymatic activity was observed at pH 3.7. This could possibly be due to the fact that the three essential histidyl residues which are coordinated to the Zn(II) ion to form the active site may be part of the seven buried histidyl residues, and protonation may result in destruction of the exact stereochemistry of the active site. The increase in intrinsic viscosity is probably a result of an expansion of the molecular domain arising from loosening of the noncovalent interactions thus permitting solvent molecules to penetrate the interior of the protein. This contention is reinforced by ultraviolet difference absorbance measurements, since a major portion of the buried aromatic residues are made accessible to solvent at this transition. The expansion also accounts for the observation on the disappearance of the aromatic CD ellipticity bands, since the expansion will probably cause the individual aromatic amino acids in the clusters, each of which presumably is located in a quite distal position in the sequence of the polypeptide chain, to move away from one another and destroy the aromatic clusters which give rise to these CD bands. The same phenomenon can also explain the changes in CD below 240 nm. Assuming that the aromatic clusters which give rise to the aromatic ellipticity bands also manifested in this region as positive ellipticity values, expansion of the molecule which destroys these asymmetric centers will cause the peptide CD bands to become more negative. Moreover, the loosening of the polypeptide backbone from expansion of the molecule will likely contribute to a more negative value of CD band in this region. The reversibility of this transition has not been fully studied. No thermodynamic reversibility is expected since a hysteresis effect on the deprotonation of the histidyl residues will likely to occur in renaturation.

The second conformational transition is distinctively separated from the first one. It is characterized by a further exposure of the buried aromatic amino acid residues to the aqueous solvent environment, a significant increase in intrinsic viscosity to 8.4 $cm^3/g$, and a decrease in magnitude of the peptide CD trough with concomitant shift to lower wavelength region which results in a decrease in the magnitude of the molar ellipticity at 222 nm. All these changes suggest a partial unfolding of the expanded molecule. The increase in viscosity is likely to be contributed by a partial unfolding of the polypeptide chain which generates a larger Stoke's radius of the molecules. The CD change of the peptide region can be attributed to the partial unfolding of the polypeptide backbone which probably converts some of the secondary structure to the disordered conformation as well as a general loosening of the molecule.

The possible complication due to the presence of the Zn(II) in the native enzyme can be ruled out since the acid denaturation of the apoenzyme gives similar results, *i.e.*, the presence of two distinct separable stages of conformational transition (K.-P. Wong and L. M. Hamlin, manuscript submitted for publication). The acid-denatured state does not seem to involve any covalent chemical modification of the protein. The acid-denatured enzyme can be converted to the random-coiled state in 6 M Gdn·HCl and then renatured to the native state with almost complete recovery of specific esterase activity and conformation as characterized by its characteristic CD spectra (K.-P. Wong and L. McCoy, unpublished results).

## Acknowledgments

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# Affinity Labeling of Rabbit Muscle Myosin with a Cobalt(III)–Adenosine Triphosphate Complex†

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ABSTRACT: Several Co(III) and Cr(III) complexes have been tested for their ability to serve as affinity labels for the active site of myosin. The complex of Co(III)–ATP and phenanthroline [Co–(phen)–ATP] was studied in detail. Its binding to myosin is stable and affects the ATPase activity; it can, however, be displaced by the addition of thiol reagents, such as dithiothreitol, with consecutive recovery of the original activity. Full labeling yields two labels per myosin molecule, which is in accordance with the existence of two "heads" per myosin molecule, each probably carrying one active site. The kinetics of labeling suggests that binding abolishes the activity of a site and that when only one of the two heads is labeled, the activity of the unlabeled one is enhanced by a factor of 3.4–3.6 under the experimental conditions. Although the label does not seem to bind to –SH groups, it protects the essential –SH groups from modification by *N*-ethylmaleimide, probably by binding in the proximity of these groups. It appears that the label is located in the region of the active site thus serving as an affinity label of the active sites of myosin.

One of the key questions in muscle contraction is the mechanism of transduction of chemical into mechanical energy. The source of chemical energy for this process is ATP which is bound and hydrolyzed by myosin. It is thus of utmost importance to elucidate the details of the interaction between myosin and ATP, and both structural (Morita, 1967; Seidel and Gergely, 1971; Werber *et al.*, 1972; Viniegra and Morales, 1972; Murphy, 1973) and kinetic (Lymn and Taylor, 1971; Bagshaw *et al.*, 1972; Schliselfeld and Kaldor, 1973) work have been directed toward this goal.

In order to determine the structure of the active site of myosin it has previously been affinity labeled. This has been performed with reagents interacting specifically with thiol groups which are known to be necessary for activity (Murphy and Morales, 1970; Yount *et al.*, 1972, 1973). We have used a different approach for affinity labeling, based on the fact that in muscle the substrate is a complex of ATP with $Mg^{2+}$. Previous studies (Danchin, 1971, 1973; Danchin and Buc, 1973; Kowalsky, 1969) have indicated the potentialities of the use of Co(III) and Cr(III) derivatives for the affinity labeling of $Mg^{2+}$, or $Mg^{2+}$–substrate, binding sites in proteins and nucleic acids. We have therefore attempted to label the active site of myosin with various Co(III) and Cr(III) complexes. At least one of these, Co(III)–phenanthroline–ATP [Co–(phen)–ATP],[1] is shown in this study to behave as an affinity label of the active sites.

## Experimental Section

Myosin and heavy meromyosin (HMM) were prepared as described by Azuma and Watanabe (1965) and by Lowey and Cohen (1962), respectively, from rabbit muscle. Both materials were stored at −18° in 50% glycerol, which was removed before use by dialysis against 5 mM phosphate buffer (pH 7.0), containing KCl (0.5 M in the case of myosin and 0.05 M in the case of HMM).

*Synthesis of Co(III)–ATP Complexes.* Co(III)–ATP, containing one molecule each of ATP and of $Co^{3+}$ per molecule, was synthesized by electrophoresis and purified in a similar manner to the Co(III)–5′AMP complex described by Danchin and Buc (1973). All other complexes were synthesized as follows. A solution containing 11 mM ATP, 10 mM $CoCl_2$, and 10 mM complexing agent was slowly brought to pH 10 (in ice) with vigorous aeration which was stopped when no color increase was observed. The mixture was then purified by two precipitations in ethanol (2:1 v/v) at −20°. This removed most of unreacted reagents.

The complexing agents were: 1,10-*o*-phenanthroline (Merck), ethylenediamine (Koch-Light), and *N*,*N*′-dimethylethylenediamine (Merck), which will hereafter be denoted as phen, en-, and dimethyl-en, respectively.

Figure 1 shows the absorption spectrum of Co–(phen)–ATP

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[1] Abbreviations used are: phen, phenanthroline; HMM, heavy meromyosin; en, ethylenediamine; dimethyl-en, dimethylethylenediamine.