Human Carbonic Anhydrase. Protein Conformation and Metal Ion Binding*

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ABSTRACT: The optical rotatory dispersion (ORD) of human carbonic anhydrase B has been determined from 800 to 190 mμ. Native Zn(II) carbonic anhydrase B has an unusual ultraviolet ORD consisting of multiple Cotton effects not characteristic of α-helical proteins. The first major trough occurs at 222 mμ, $[\alpha]_{222}$ − 2650°, followed by a small positive rotation at 205 mμ, $[\alpha]_{205}$ +500°, and a large trough at 195 mμ, $[\alpha]_{195}$ −5400°. In addition, there are several Cotton effects in the region of the aromatic absorption bands with prominent maxima at 292 and 265 mμ. Below pH 4.0, the ORD of Zn(II) carbonic anhydrase shows a radical change accompanied by the *irreversible* loss of zinc and activity. At pH 1.9, the ORD curve has the general features expected of a protein containing areas of α-helix: trough at approximately 233 mμ, $[\alpha]_{233}$ −5150°, a shoulder in the region of 215 mμ, and the peak of a large positive Cotton effect at 197 mμ, $[\alpha]_{197}$ +12,200°. The aromatic Cotton effects disappear. Apocarbonic anhydrase B at pH 7.5 has an ORD curve very similar to the native zinc enzyme including the aromatic Cotton effects. However, the acid-induced change in ORD occurs at higher pH values and can be reversed by Zn(II) ions. Zn(II), Co(II), Ni(II), Cu(II), Cd(II), and Hg(II) carbonic anhydrases have very similar ultraviolet ORD curves. Although Co(II) carbonic anhydrase has additional visible absorption bands at 520 mμ (ε 205), 555 (340), 615 (230), and 640 (240), these are not optically active.

Combination of the cobalt enzyme with acetazolamide causes an intensification and red shift of the major absorption band, maximum at 580 mμ (ε 540), and a decrease in the band splitting. This is accompanied by the appearance of asymmetric positive anomalous rotatory dispersion centered at 554 mμ with an amplitude, [M] = 4500°. CN^- causes a similar shift in spectrum and the appearance of anomalous rotatory dispersion, [M] = 3000°. HCO_3^- induces marked changes in the spectrum of cobalt carbonic anhydrase, but only a slight perturbation in the ORD curve. No changes occur in the spectrum or ORD of the acetazolamide-inhibited enzyme upon the addition of HCO_3^-, implying that bicarbonate combines near the cobalt ion in the uninhibited enzyme. Cu(II) carbonic anhydrase has a broad asymmetric absorption band with a maximum at 750 mμ (ε 100). This band is also optically inactive. However, addition of CN^- intensifies and shifts the maximum to 700 mμ (ε 130) accompanied by the appearance of a positive Cotton effect centered at 700 mμ, [M] = 5000°. A model for the geometry of the metal complex at the active center of carbonic anhydrase is proposed.

Zinc metalloenzymes have received increasing interest because of the distinctive chemical and enzymatic features resulting from the presence of the metal ion (Vallee, 1959, 1964). Special attention has been given to carboxypeptidase A from bovine pancreas and the carbonic anhydrases from human and bovine erythrocytes, since with these enzymes it has been possible to prepare stable inactive zinc-free apoenzymes to which activity can be fully restored by readdition of the metal ion (Vallee *et al.*, 1960; Lindskog and Malmström, 1962; Rickli and Edsall, 1962).

This circumstance has led to certain analogies between the metal–protein equilibria in these metalloenzymes and the metal–ligand equilibria observed in model coordination complexes (Coleman and Vallee, 1961; Vallee and Coleman, 1964; Lindskog and Nyman, 1964). Both proteins lose the metal ion with increasing hydrogen ion concentration and in the presence of competing chelating agents such as 1,10-phenanthroline. The equilibria have been treated quantitatively by the pH–pM relationships established for model chelate systems, and the stability constants have been determined for a series of first transition and II-B metallocarboxypeptidases (Coleman and Vallee, 1961) and metallocarbonic anhydrases (Lindskog and Nyman, 1964). The validity of comparing such data with those obtained in model metal–ligand systems depends on the absence of major change in protein three-dimensional structure and metal-binding site accompanying metal ion removal, an assumption supported in the case of carboxypeptidase A by X-ray diffraction data (Rupley and Neurath, 1960).

The present study was undertaken to investigate the possible influence of the metal ion on the three-dimensional structure of human carbonic anhydrase B as

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reflected in the ultraviolet optical rotatory dispersion. An additional parameter for the study of the protein structure in the immediate vicinity of the metal ion is provided by the optical activity of the visible absorption bands introduced into the protein by the substitution of cobalt and copper for zinc. The anomalous rotatory dispersion of these bands provides information on the geometry of the metal ion complex at the active center of carbonic anhydrase and on the changes in geometry which accompany inhibitor and substrate binding.

Materials and Methods

Carbonic anhydrase was prepared from pooled human erythrocytes by a procedure similar to that reported by Rickli *et al.* (1964). Washed packed red cells were hemolyzed by addition of equal volumes of cold deionized water, and the hemolysate was buffered to approximately pH 6.8 with the required amounts of solid Na_2HPO_4 and NaH_2PO_4 to give a final concentration of 0.2 M. Separation of carbonic anhydrase from hemoglobin was accomplished by applying 50-ml aliquots of the hemolysate to 4 × 150 cm columns of G-75 Sephadex (bead form) and eluting with 0.2 M sodium phosphate buffer, pH 6.8. The fractions containing the carbonic anhydrase activity were pooled, dialyzed against metal-free water, lyophilized, and redissolved in a small volume of 0.009 M phosphate buffer, pH 6.8. This material was applied to 20 × 140 mm columns of hydroxylapatite (Tisèlius *et al.*, 1956) and eluted with a linear gradient of phosphate buffer from 0.009 to 0.2 M. Carbonic anhydrase emerged as two peaks, a major one with a specific activity of 10,000 units previously named carbonic anhydrase B, and a minor peak with a specific activity of 30,000 units previously named carbonic anhydrase C (Rickli *et al.*, 1964). The experiments to be described in this paper were all performed with carbonic anhydrase B. The preparation (5.9 mg/ml) was homogeneous in the ultracentrifuge at pH 6.8, 0.1 M phosphate buffer, with an $s_{20,w}$ of 3.18 S. The preparation contained 1 g-atom of zinc/mole assuming a molecular weight of 30,000 (Rickli *et al.*, 1964).

Apocarbonic anhydrase was prepared by dialyzing the zinc enzyme, 2–6 × 10^{-4} M, against a 500-fold volume excess of 4 × 10^{-3} M 1,10-phenanthroline contained in 0.01 M sodium acetate buffer, pH 5.5. After 15 days the zinc content of the enzyme was less than 5% of the original value, and the preparation had less than 5% of the original activity.

Cobalt, nickel, copper, cadmium, and mercury carbonic anhydrases were prepared by dialyzing aliquots of the apoenzyme against a 100-fold volume excess of 1 × 10^{-4} M Me^{2+} in 0.025 M Tris, pH 8.0. All metal solutions were prepared from the spectrographically pure metals or their salts (Johnson Mattehey Company, Ltd.).

Carbonic anhydrase activity was determined by the colorimetric procedure of Wilbur and Anderson (1948) employing a reaction mixture containing 2 ml of 0.025 M Veronal buffer, pH 8.2, and 1 mg % of brom thymol blue, 4°. Enzyme solution (1 ml) was added, and 2 ml of a cold saturated solution of CO_2 was injected by a syringe. Activity units are defined by the following formula

$$U = 10(T_b/T_c - 1)/\text{mg of protein}$$

where T_b is the time of the uncatalyzed reaction (color change of the indicator from blue to yellow-green) and T_c is the time for the enzyme-catalyzed reaction.

Chemicals were all reagent grade. All buffers were made metal free by extraction with a CCl_4 solution of dithizone (Vallee and Gibson, 1948). Brom thymol blue was made metal free by passage over Chelex resin (Bio-Rad Laboratories), and all glassware was acid cleaned by techniques described previously (Thiers, 1957). Metal-free HCl was prepared from hydrogen chloride gas and NaOH was freed of metal ions by passage over Chelex resin. Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide, Diamox) was kindly supplied by Dr. E. H. Dearborn of the Lederle Research Laboratories.

^{65}Zn and ^{60}Co binding to carbonic anhydrase was measured by the equilibrium dialysis technique of Coleman and Vallee (1960) employing a Tri-Carb scintillation spectrometer Model 3002 (Packard Instrument Co.). The labeled enzymes were prepared from apocarbonic anhydrase.

Protein concentrations were measured either by 10% trichloroacetic acid precipitation followed by drying at 104° (Hoch and Vallee, 1953) or from absorbance at 280 mμ employing a molar absorptivity of 4.9 × 10^4 M^{-1} cm^{-1} (Rickli *et al.*, 1964).

Absorption spectra were obtained with a Zeiss PM-QII spectrophotometer or with a Bausch and Lomb recording spectrophotometer (Model 515). Detailed studies of the visible absorption spectra of the metallocarbonic anhydrases were performed with a Perkin-Elmer Model 350 recording spectrophotometer equipped with a scale expander.

Optical rotatory dispersion (ORD) measurements were made from 800 to 190 mμ with a Cary Model 60 recording spectropolarimeter. A water-jacketed cell of 10-mm length with removable quartz windows was employed throughout. All measurements were made at 27° except where indicated in the text. Protein concentrations varied from 0.03 to 20 mg/ml depending on the rotatory power of the particular band being investigated. The slit width was programmed to give a constant light intensity at all wavelengths. In view of differences in the performance of various Cary Model 60 spectropolarimeters, it should be noted that slit widths in the visible region were 0.5 mm or less out to 725 mμ. Measurements to 750 mμ required opening the slit width to 1 mm, while measurements to 800 mμ required slit widths up to 2.0 mm (dynode voltage below 0.4 kv) or 1.0 mm (dynode voltage below 0.6 kv). The 800-mμ band width at the latter slit openings is 50–100 mμ; however, this is still within the band width of the copper absorption bands which are several hundred millimicrons. Once the base line for the cell assembly and blank was established, a set of measurements was performed without dismantling the cell assembly be-

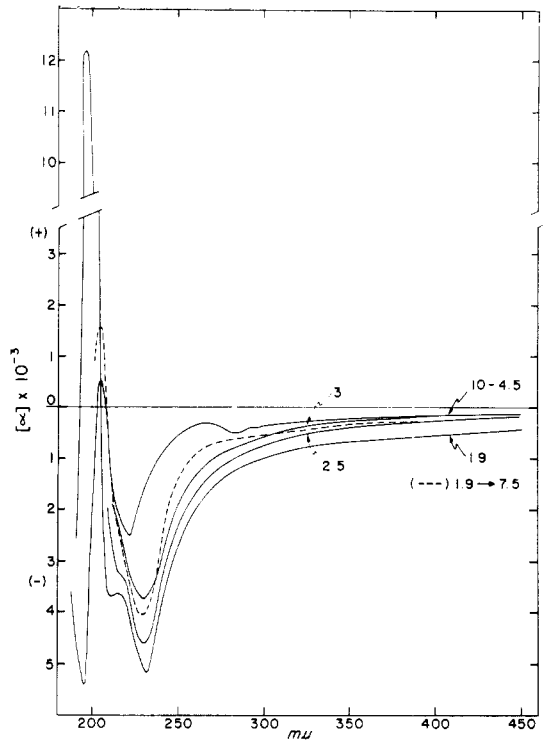


FIGURE 1: Ultraviolet ORD of carbonic anhydrase B: pH dependence. Concentrations of protein varied from 2×10^{-6} M for the far-ultraviolet determinations to 5×10^{-5} M for the near-ultraviolet determinations. Carbonic anhydrase was dissolved in 0.025 M Tris–0.025 M sodium acetate, at the pH values indicated on the figure; - - - -, enzyme incubated at pH 1.9 for 2 hr and then readjusted to pH 7.5.

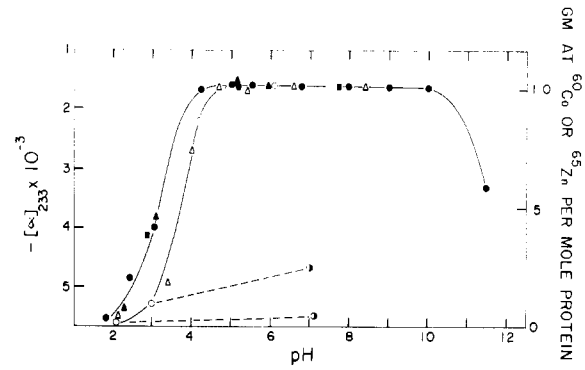


FIGURE 2: Comparison of specific rotation at 233 mμ, ^{65}Zn(II) binding, and ^{60}Co(II) binding of carbonic anhydrase B as a function of pH. ●, $[\alpha]_{233}$ of Zn(II) carbonic anhydrase; ▲, $[\alpha]_{233}$ of Co(II) carbonic anhydrase; ○, ^{65}Zn(II) bound to the protein, g-atom per mole; △, ^{60}Co(II) bound to the protein, g-atom per mole; ◑, ^{65}Zn(II), nondialyzable, rebound to protein after exposure to pH 2.0 or pH 3.0; ■, $[\alpha]_{233}$ of Hg(II) carbonic anhydrase. All protein solutions were 1×10^{-5} M contained in 0.025 M Tris–0.025 M sodium acetate, 23°.

tween samples. This procedure resulted in very reproducible base lines. All measurements were carried out with nitrogen flushing of the lamp housing and monochromator. For measurements below 220 mμ the polarimeter was also flushed with nitrogen.

Rotation is expressed as specific rotation, [α], or molar rotation, [M] = [α] × mol wt/100. In the visible wavelength region measurements of [α] are ±1°; in the ultraviolet down to approximately 220 mμ, ±20°; and below 220 mμ there is an increase to ±300°.

Results

The ultraviolet ORD curve from 450 to 190 mμ for native zinc carbonic anhydrase B is shown in Figure 1. The first major trough of a negative Cotton effect comes at 222 mμ, $[\alpha]_{222}$ −2650°, with a crossover point at about 208 mμ, a small positive excursion with a maximum at the turnover point of 500°, and the trough of a large negative Cotton effect at approximately 195 mμ, $[\alpha]_{195}$ −5400°. In addition, the area 250–300 mμ shows anomalous rotatory dispersion of relatively small amplitude with prominent maxima located at 265 and 292 mμ.

Carbonic anhydrase is known to undergo rather large changes in rotation above 300 mμ at hydrogen ion concentrations greater than 10^{-4} M (Rickli *et al.*, 1964), accompanied by considerable change in the aromatic absorption bands (Rickli *et al.*, 1964; Riddiford, 1965). Furthermore, removal or exchange of the zinc atom in carbonic anhydrase has been very difficult except at low pH values (Keilin and Mann, 1940; Day and Franklin, 1946; Tupper *et al.*, 1951; Lindskog and Malmström, 1962; Rickli and Edsall, 1963). For this reason, the ORD of carbonic anhydrase from 450 to 190 mμ was determined as a function of pH (Figure 1) and compared to ^{65}Zn binding as a function of pH (Figure 2).

As the pH is lowered, the ORD remains identical with that of the enzyme at pH 7.5 until about pH 4.0. Below pH 4.0, there is a very sharp increase in levorotation in the near-ultraviolet, a disappearance of the anomalous dispersion in the region 250–300 mμ, and a shift of the trough of the first negative Cotton effect from 222 mμ to approximately 233 mμ. In addition, the first minimum increases from an [α] of −2650° to −5150° (Figure 1). The most striking change is the appearance of a very large positive Cotton effect with a peak at 197 mμ, $[\alpha]_{197}$ +12,200°, and a second crossover point at 193 mμ. There also appears a shoulder in the region of 215 mμ.

The ORD change upon acidification is accompanied by the appearance of an ultraviolet difference spectrum identical with that reported by Rickli *et al.* (1964) and Riddiford (1965). A similar change takes place in the ORD curve if the pH exceeds 11.0, as indicated by $[\alpha]_{233}$ in Figure 2. Urea (8 M) also induces similar ORD changes and ultraviolet difference spectra. The acid-

induced change in the ORD profile is partially reversible. If the sample is taken to pH 1.9 for 2 hr and then readjusted to pH 7.5, [α] becomes considerably less negative in the near-ultraviolet, the first trough shifts back to about 227 mμ, $[\alpha]_{227}$ −4000°, and the large peak at 197 mμ disappears. While no definite aromatic Cotton effects reappear, the greatest reversal of [α] in the near-ultraviolet takes place in the 250–300 mμ region.

The specific rotation at 233 mμ as a function of pH is plotted in Figure 2 together with ^{65}Zn binding to the enzyme as a function of pH. ^{65}Zn is firmly bound to the enzyme until the acid-induced conformational change starts and then is rapidly released. The steeper loss of ^{65}Zn reflects the fact that the dialysis procedure requires at least several hours for the dialysis bags to equilibrate. However, once the protein is below the pH where the conformational change begins, there is a continuing slow denaturation as indicated by a drift in the rotation to more negative values. Hence, the true equilibrium point is difficult to determine. The rotation changes plotted are those occurring immediately after adjustment of the pH to the stated value.

Activity is lost at pH 2.0 and is not restored to the protein by returning the pH to 7.5 (Table I). Likewise,

TABLE I: Activities and Zinc Content of Human Metallocarbonic Anhydrases B.

Metal Enzyme	Activity (U)	Zinc Content (g-atom/mole)
Zn(II) native	10,200	1.0[a]
Zn(II) native + acetazolamide (1×10^{-5} M)	187	...
Zn(II) native, pH 1.9 → pH 7.5	0	...
Apoenzyme	400	0.03[a,b]
Zn(II) + apoenzyme	9,500	1.0[b]
Co(II) + apoenzyme	5,700	0.05[a]
Ni(II) + apoenzyme	500	0.05[a]
Cu(II) + apoenzyme	127	<0.01[a]
Cd(II) + apoenzyme	430	<0.01[a]
Hg(II) + apoenzyme	5	<0.01[a]

[a] Determined by a dithizone method (Vallee and Gibson, 1948). [b] Determined by ^{65}Zn content.

specific ^{65}Zn-binding is not restored to the acid-denatured protein on return to neutral pH, and the zinc remains freely dialyzable (Figure 2).

The binding of $^{60}Co(II)$ to carbonic anhydrase B has the same pH dependence as $^{65}Zn(II)$ binding (Figure 2). Cobalt, like zinc, does not dissociate from the protein until the acid conformational change begins. The acid

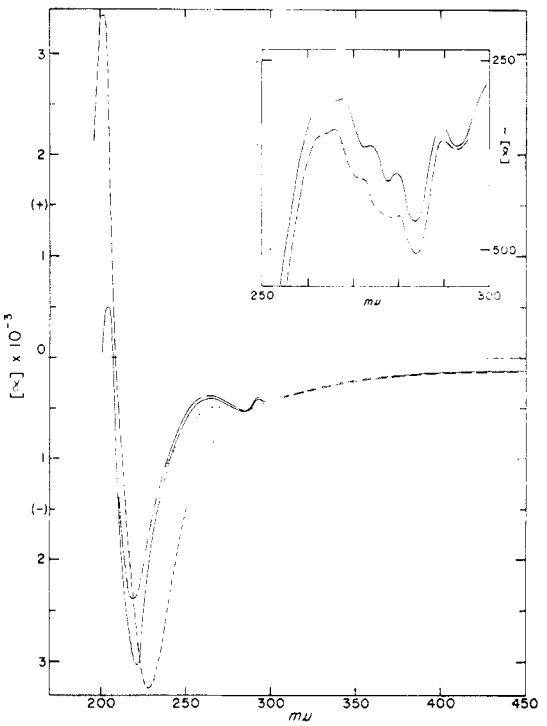


FIGURE 3: Ultraviolet ORD of apocarbonic anhydrase B. ———, apocarbonic anhydrase, pH 7.5 in 0.025 M Tris, 27°; solid line broken at 250 mμ, apocarbonic anhydrase, pH 7.5 plus 2 molar excess of Zn(II), pH 7.5 in 0.025 M Tris; – – – –, apocarbonic anhydrase, pH 5.5 in water, 27°; ·····, apocarbonic anhydrase, pH 5.5 plus 2 molar excess of Zn(II) in 0.025 M sodium acetate, pH 5.5. Below 240 mμ, —·— represents both proteins to which Zn(II) has been added. Protein concentrations were as described in Figure 1. Insert: Detail of the aromatic region. ———, zinc carbonic anhydrase; – – – –, apocarbonic anhydrase. Protein solutions were 6×10^{-5} M contained in 0.025 M Tris, pH 7.5, 27°.

conformational changes in mercury carbonic anhydrase, identical with those of the zinc enzyme, are also indicated in Figure 2 by $[\alpha]_{233}$ at pH 7.5, 5.0, and 2.8.

Apocarbonic anhydrase prepared at pH 5.5 and readjusted to pH 7.5 by dialysis against metal-free buffer has an ORD curve very similar to the native zinc enzyme with the exception that the first major minimum consistently shows greater negative rotation, $[\alpha]_{222}$ −3000° (Figure 3). The position and profiles of the aromatic Cotton effects remain the same as those observed in the zinc enzyme at pH 7.5. Even the small details are quite similar. As has been noted previously (Lindskog and Nyman, 1964), the above procedure for preparing the apoenzyme is associated with a moderate amount of precipitation when the apoenzyme solution is returned to pH 7.5; this material is first removed by centrifugation.

If the pH is readjusted to 5.5, however, the rotation of the apoenzyme in the region 300–200 mμ becomes

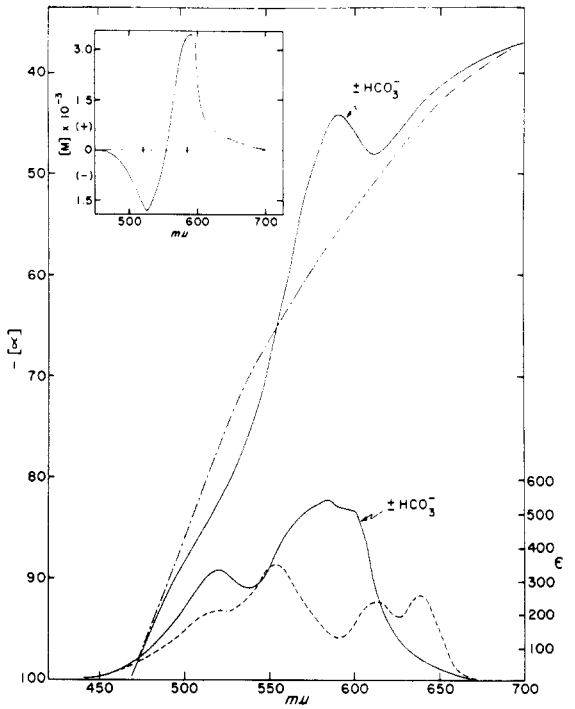


FIGURE 4: Effect of acetazolamide on the visible ORD and spectrum of cobalt carbonic anhydrase B. ORD curve: —·—, zinc carbonic anhydrase and cobalt carbonic anhydrase; ———, cobalt carbonic anhydrase plus acetazolamide (an identical curve is obtained in the presence of 0.5 M HCO_3^-). Insert: Difference ORD, ($[M]_\lambda$ for cobalt carbonic anhydrase + acetazolamide) − ($[M]_\lambda$ for cobalt carbonic anhydrase). Lines on the zero abscissa indicate the positions of the absorption maxima of the inhibited enzyme. Spectra: - - - -, cobalt carbonic anhydrase; ———, cobalt carbonic anhydrase plus acetazolamide (an identical curve is obtained in the presence of 0.5 M HCO_3^-). Both sets of data were obtained on a 2×10^{-4} M solution of cobalt or zinc carbonic anhydrase in 0.025 M Tris, pH 9.0, 27°. Acetazolamide was added in a concentration of 4×10^{-4} M. ϵ = molar absorptivity, $M^{-1}\,cm^{-1}$.

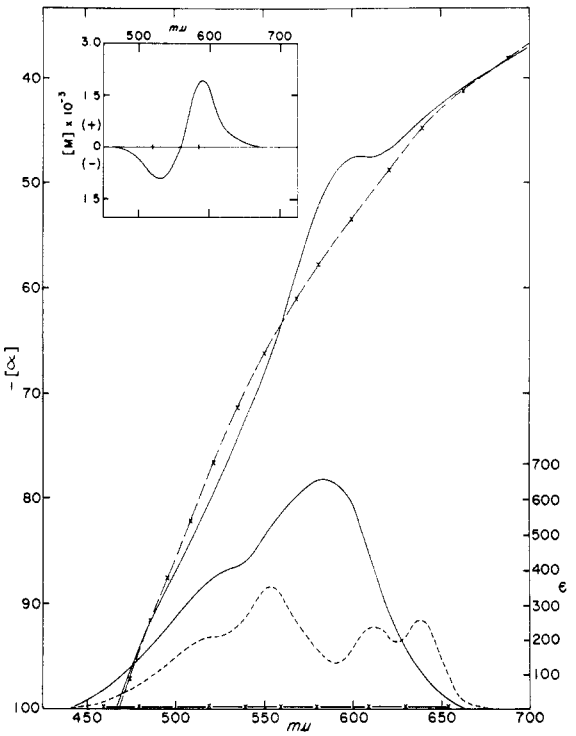


FIGURE 5: Effect of CN^- on the visible ORD and spectrum of cobalt carbonic anhydrase B. ORD curves: - - - -, cobalt carbonic anhydrase; ———, cobalt carbonic anhydrase plus 5 molar excess of CN^-; ×—×, cobalt carbonic anhydrase plus 100 molar excess of CN^-. Insert: Difference ORD, ($[M]_\lambda$ for cobalt carbonic anhydrase + CN^-) − ($[M]_\lambda$ for cobalt carbonic anhydrase). Lines on zero abscissa indicate the positions of the absorption maxima of CN^--inhibited cobalt carbonic anhydrase. Spectra: - - - -, cobalt carbonic anhydrase; ———, cobalt carbonic anhydrase plus 5 molar excess of CN^-; ×—×, cobalt carbonic anhydrase plus 100 molar excess of CN^-. All data were obtained on a 2×10^{-4} M solution of cobalt carbonic anhydrase in 0.025 M Tris, pH 9.0, 27°. ϵ = molar absorptivity, $M^{-1}\,cm^{-1}$.

markedly more negative; the aromatic Cotton effects disappear except for a slight perturbation, and the first minimum shifts to 228 mμ and becomes more negative, $[\alpha]_{228}$ −3300° (Figure 3). In addition, the rotation becomes much more positive around 200 mμ, $[\alpha]_{202}$ +3400°. Addition of equimolar Zn(II) ions to the apoenzyme at pH 5.5 results in the reappearance of the aromatic Cotton effects, the return of the first minimum to 222 mμ, $[\alpha]_{222}$ −2600°, identical with that for the native zinc enzyme (Figure 3). If 24 hr are allowed to elapse between the time of adjustment of the apoenzyme solution to pH 5.5 and the readdition of Zn(II) ions, the return of the aromatic Cotton effects becomes less complete.

Ultraviolet ORD curves from 450 to 190 mμ for Co(II), Ni(II), Cu(II), Zn(II), Cd(II), and Hg(II) carbonic anhydrase B are almost identical except for small but reproducible deviations in the larger of the aromatic Cotton effects and in the depth of the trough at 222 mμ. These differences can be summarized by the observation that Zn(II), Cd(II), Hg(II), and Cu(II) carbonic anhydrase all have slightly higher peaks at 265 ± 5 mμ than Co(II) and Ni(II), while the latter have a slightly deeper trough at 222 mμ.

None of the first transition or other II-B metallocarbonic anhydrases bind $^{65}Zn(II)$, implying that the zinc-binding site is occupied by these metal ions. Detectable exchange at pH 7.5 as followed by the binding of $^{65}Zn(II)$ requires several weeks. More rapid exchange can be achieved at pH 5.5; *e.g.*, $^{65}Zn(II)$ exchanges for enzyme-bound $^{60}Co(II)$ with a half-time

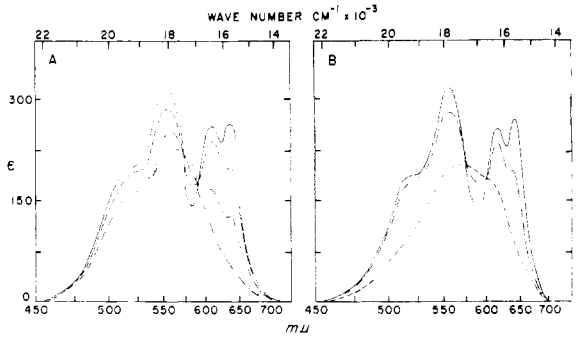


FIGURE 6: Effect of HCO_3^- and H^+ on the visible absorption spectrum of cobalt carbonic anhydrase B. A: Effect of HCO_3^-: ———, cobalt carbonic anhydrase, pH 8.6; plus 0.02 M HCO_3^- (– – – –); plus 0.25 M HCO_3^- (— · —); plus 0.3 M HCO_3^- (·····); plus 0.5 M HCO_3^- (— · · —). Bicarbonate was added to the cuvet as solid $NaHCO_3$ in amounts to give the final concentrations noted. The cuvet was inverted for mixing and the spectrum taken within 1 min. Initial pH was 8.6, final pH was 8.2. Protein concentration was 2×10^{-4} M in 0.025 M Tris, 27°. B: Effect of H^+: ———, cobalt carbonic anhydrase, pH 9.0; — – —, cobalt carbonic anhydrase, pH 7.5; – – – –, cobalt carbonic anhydrase, pH 5.5. Protein concentration was 2×10^{-4} M in 0.025 M Tris–0.025 M sodium acetate, 27°; ϵ = molar absorptivity, M^{-1} cm^{-1}.

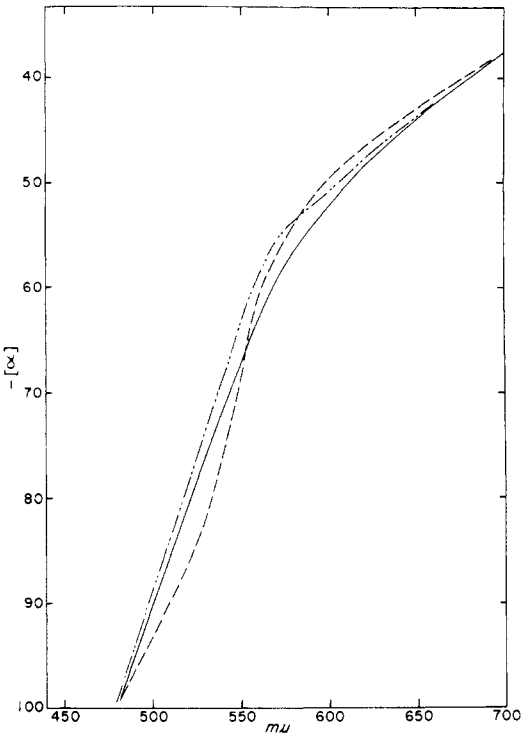


FIGURE 7: Effect of HCO_3^- and H^+ on the visible ORD of cobalt carbonic anhydrase B. ———, cobalt carbonic anhydrase, pH 9.0; — · · —, cobalt carbonic anhydrase plus 0.5 M HCO_3^-, pH 8.2; – – – –. cobalt carbonic anhydrase, pH 5.5. Conditions are as in Figure 6.

of approximately 10 days. The total amount of enzyme-bound isotope during the experiment remains 1 g-atom/mole, also implying that the same site is occupied by the two ions. X-Ray diffraction data used to construct electron density difference maps of crystalline carbonic anhydrase have located the Zn(II)- and Hg(II)-binding sites in the unit cell, and they are identical within 1–2 A (Malmström *et al.*, 1964). Only Zn(II) and Co(II) carbonic anhydrase B show significant catalysis of the hydration of CO_2 (Table I), in agreement with the data of Lindskog and Nyman (1964).

In the region 450–700 mμ, native zinc carbonic anhydrase has no absorption maxima and a plain ORD curve (Figure 4). Substitution of Co(II) for Zn(II) at the active site of carbonic anhydrase is accompanied by the appearance of multiple absorption maxima in the region 500–650 mμ, reflecting the d–d electronic transitions of the cobalt ion. These bands have maxima at 520 mμ (ϵ 205), 555 (340), 615 (230), and 640 (240). The values are in close agreement to those reported by Lindskog and Nyman (1964) for human cobalt carbonic anhydrase B at pH 9.0. None of these absorption bands have measurable optical activity. The ORD curve of the protein remains plain and identical with that observed for the zinc enzyme.

Binding of the sulfonamide inhibitor, acetazolamide, to the cobalt enzyme is accompanied by considerable intensification of the spectrum and a red shift in the major absorption maximum (Figure 4). The two main maxima of the cobalt–enzyme–acetazolamide complex are located at 520 mμ (ϵ 330) and 580 mμ (ϵ 540) with a shoulder in the region 590–600 mμ (ϵ 505). These bands are now optically active, generating asymmetrical anomalous rotatory dispersion with a peak at 590 mμ, a crossover point (compared to the uninhibited enzyme) at 554 mμ, and a trough at 525 mμ. Although the total amplitude when expressed as [α] for a complex with a molecular weight of 30,000 is only 15°, the molar rotation of these bands is considerable, approximately 4500°. Addition of 0.5 M HCO_3^- to the inhibited enzyme alters neither the absorption spectrum nor the anomalous rotatory dispersion.

Similar spectral shifts and anomalous rotatory dispersion are induced by the addition of a 1:1 molar ratio of CN^- to the cobalt enzyme (Figure 5). There is very little intensification of the spectrum on increasing the ratio to 4:1 or 5:1. The main absorption maximum of the cyanide complex is located at 585 mμ (ϵ 650), with a prominent shoulder at 520 mμ. The anomalous rotatory dispersion is very similar in shape to that induced by the sulfonamide, but of less rotatory power. Total molar rotation is about 3000°. The peak is located at 590 mμ, crossover at 560 mμ, and trough at 530 mμ.

An increase of the cyanide:enzyme ratio to 100:1 results in bleaching of the spectrum and return of the ORD curve to that observed in the native enzyme (Figure 5). Cobalt is simultaneously removed from the

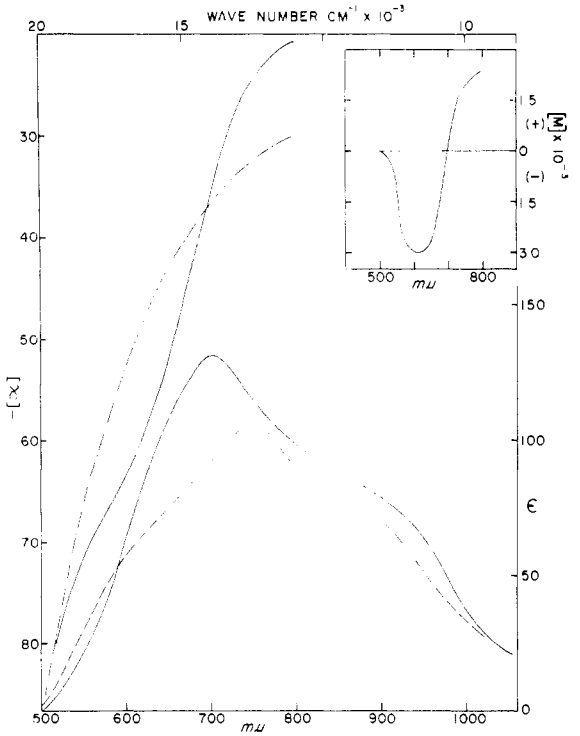


FIGURE 8: Effect of CN^- on the visible ORD and spectrum of copper carbonic anhydrase B. ORD curves: —·—, zinc carbonic anhydrase and copper carbonic anhydrase; ———, copper carbonic anhydrase plus 10 molar excess of CN^-. Insert: Difference ORD, ($[M]_\lambda$ for copper carbonic anhydrase + CN^-) − ($[M]_\lambda$ for copper carbonic anhydrase). Spectra: - - - -, copper carbonic anhydrase; ———, copper carbonic anhydrase plus 10 molar excess of CN^-. All data were obtained on a 6×10^{-4} M solution of copper or zinc carbonic anhydrase in 0.001 M sodium acetate, pH 7.0. ϵ = molar absorptivity, $M^{-1}\ cm^{-1}$.

enzyme, as demonstrated by dialyzing a ^{60}Co-labeled enzyme against a 100-fold molar excess of cyanide.

While addition of HCO_3^- to the sulfonamide-*inhibited* cobalt enzyme has no effect on the absorption spectrum, addition of increasing increments of HCO_3^- to the *uninhibited* cobalt enzyme results in an immediate and marked change in the spectrum (Figure 6A). The long wavelength maxima at 615 and 640 mμ disappear and the main absorption maximum decreases in intensity by about 25%, accompanied by a slight shift to longer wavelength. Initial and final pH readings accompanying the addition of solid $NaHCO_3$ to the cuvet were 8.6 and 8.2, respectively.

These changes are very similar to those occurring when cobalt carbonic anhydrase is titrated to pH 5.5 as previously reported by Lindskog and Nyman (1964), and repeated here with almost identical results (Figure 6B). An essential feature for the interpretation of the latter spectrum is the demonstration that cobalt is not lost from the enzyme over this pH range (Figure 2).

In marked contrast to the spectral changes induced by the inhibitors, the spectral changes induced by HCO_3^- and H^+ are accompanied by only minor changes in ORD (Figure 7). Acidification to pH 5.5 induces an almost symmetrical positive Cotton effect of very low amplitude, about 5°, while 0.5 M HCO_3^- induces a positive parturbation of 2–3° with a peak in the region of 560 mμ. Both the spectral and ORD changes are completely reversible by rapid dialysis of the respective enzyme solutions against metal-free buffer at pH 9.0.

Addition of acetazolamide, CN^-, or HCO_3^- to the zinc or cobalt enzymes causes no alterations in the ultraviolet ORD down to 210 mμ. Acetazolamide has an absorption maximum at 272 mμ (ε 7850). Under conditions where the inhibitor is 95% bound (Table I) no shift occurs in the absorption maximum of the inhibitor.

Copper carbonic anhydrase has a broad asymmetric absorption band in the region 500–1000 mμ with a maximum at 750 mμ (ε 100) (Figure 8). The spectrum is similar to that reported by Lindskog and Nyman (1964) for copper carbonic anhydrase B. At the concentration of protein employed (18 mg/ml) and over a 1-cm path length, the ORD up to 800 mμ is not significantly different from that observed for the zinc enzyme at the same concentration. Addition of cyanide to the copper enzyme intensifies the band, and the major maximum shifts to 700 mμ (ε 130). The specific rotation at 800 mμ becomes ~10° more positive and the negative arm of a positive Cotton effect appears below 700 mμ. The anomalous dispersion is centered at the position of the major absorption band, and the total molar rotation is approximately 5000°. Both the spectral and ORD changes do not become complete until a 10-fold molar excess of CN^- is added.

Discussion

Native zinc carbonic anhydrase B has an ORD which consists of multiple Cotton effects not characteristic of globular proteins containing α-helical segments. The first major trough occurs at 222 mμ rather than 233 mμ, and the trough of a large negative Cotton effect appears at 195 mμ in place of the peak of a large positive Cotton effect (Figure 1). In addition to these far-ultraviolet Cotton effects, there are superimposed Cotton effects attributable to the 250–300 mμ absorption bands (Figure 1 and 3). The portion of this curve above 220 mμ for the human B and C enzymes has been reported recently by Myers and Edsall (1965), who have described the aromatic Cotton effects in detail. It is difficult to say precisely what structure is indicated by the ORD curve for native carbonic anhydrase, since the Cotton effects observed for structures of polypeptide chains other than the α-helix and random coil are not well characterized (Schellman and Schellman, 1964). The positions of the maxima and minima may reflect Cotton effects of the chromophoric side chains superimposed on those of the peptide backbone.

While the rotatory power reported for aromatic

absorption bands in amino acids and polypeptides would not seem sufficient for a few residues to make a prominent contribution to protein rotation (Iizuka and Yang, 1964; Fasman *et al.*, 1964), it seems quite probable that in the proper environment the rotatory power of given aromatic transitions could be considerably enhanced. Thus any or all of the 8 tyrosine, 6 tryptophan, and 10 phenylalanine residues of carbonic anhydrase B may contribute to the anomalous dispersion in the aromatic region which appears quite rich in detail (Figure 3). There are several indications that most of the tyrosines of carbonic anhydrase are unusual. There is evidence for only 1 normally titrating tyrosine in the titration curve of the undenatured protein (Riddiford, 1964). In addition, none of the tyrosines in native carbonic anhydrase react with acetylimidazole (Coleman, unpublished data) under conditions where this reagent has been shown to react readily with free tyrosines in a variety of proteins (Simpson *et al.*, 1963; Wacker *et al.*, 1964).

The possibility of a major contribution to the trough at 222 mμ from Cotton effects due to the aromatic or other chromophoric side chains cannot be ruled out. It would seem safe to associate the negative Cotton effect at 195 mμ with the peptide bond transitions. Polypeptides and proteins are known which show both well-developed aromatic Cotton effects and the large trough in the region 233–238 mμ thought characteristic of the α-helix (Fasman *et al.*, 1964; Beychok and Fasman, 1964; Ulmer, 1965). Thus, from the ORD of native carbonic anhydrase it is difficult to visualize a contribution from either of the Cotton effects usually associated with the right-handed α-helix.

Although the metal ion is not essential to the maintenance of the structure giving rise to the aromatic Cotton effects, it has a marked effect on the pH stability of this structure (Figure 3). By pH 5.5 the aromatic Cotton effects of the apoenzyme have largely disappeared in contrast to the zinc enzyme in which they are present unchanged at pH 5.5. The stabilizing effect of the metal ion on the aromatic structure is emphasized by the ability of Zn^{2+} ions added at pH 5.5 to restore partially the aromatic Cotton effects. This does not of course imply that the aromatic residues contribute the donor atoms to the metal-binding site, although tyrosyl hydroxyls could function in this capacity. There are, however, forces in addition to those provided by chelation of the metal ion which stabilize this structure, since the aromatic Cotton effects are present in the absence of the metal ion at pH 7.5 (Figure 3).

The accompanying deepening and red shift of the first major trough and increase in positive rotation in the 200–205 mμ region, both completely reversed by the addition of Zn^{2+} ions, imply that the reversible transformation in the apoenzyme between pH 7.5 and pH 5.5 may also be accompanied by moderate rearrangement of general protein structure.

Thus, at pH 5.5 there is present a strong tendency for rearrangement of the protein structure which would explain the ease of removal of the metal ion at this pH value. At pH 5.5 this conformational change is largely reversible, so that an active zinc enzyme can be reconstituted (Table I). If the apoenzyme, especially in dilute solution, is allowed to stand for 24 hr or more at pH 5.5, the conformational change becomes less reversible, which probably accounts for the invariable presence of precipitated protein in the apoenzyme preparations.

Increase in hydrogen ion concentration is associated with metal ion release in a number of metalloproteins. In some cases, *e.g.*, yeast alcohol dehydrogenase, this seems to be accompanied by irreversible change in protein structure (Kägi and Vallee, 1960), while in others, *e.g.*, carboxypeptidase A, there seems to be no change in structure of the apoenzyme as a function of pH and complete reversibility is possible (Vallee *et al.*, 1960; Rupley and Neurath, 1960). Carbonic anhydrase appears to fall in between these two extremes.

The enzyme maintains its native structure and zinc content until about pH 4.0 at which point there is a rather sharp transition to quite a different ORD curve, and simultaneously the zinc is lost. The difference in specific rotation at 195 mμ of about +15,000° between the native and acid-treated protein (Figure 1) would indicate that this change is associated with a change in the nature of the asymmetry of a large proportion of the peptide bonds. By the criteria that have recently been employed for assessing the α-helical content of proteins (Schellman and Schellman, 1964; Harrison and Blout, 1965), the acid-treated carbonic anhydrase would qualify as containing areas of α-helix. The possibility of α-helix appearing in the acid-treated protein was first suggested by Rickli *et al.* (1964) on the basis of the calculation of a more negative b_0 value for the near-ultraviolet acid rotatory dispersion curve and also on the hypochromicity appearing in the far-ultraviolet absorption bands. There is perhaps precedent for this in the fact that poly-L-glutamic acid assumes the random form at neutral pH while it assumes the α-helical form at pH 4.3.

Although [α] becomes markedly less negative throughout the near-ultraviolet region on return to neutral pH, indicating some reversibility of the acid-induced conformational change, the native structure does not seem to re-form readily (Figure 1). The relatively greater reversal of [α] in the area of the previous aromatic Cotton effects would seem to imply that a structure is assumed in which the rotatory power of the aromatic transitions is again enhanced.

The native structure is essential for the maintenance of the zinc-binding site and activity, since both are lost irreversibly with acid (Figure 2, Table I). However, in contrast to the less drastic conformational change in the apoenzyme at pH 5.5, the metal ion apparently has little influence on the transformation in the molecule below pH 4.0, as is emphasized by the failure of the pH dependence of metal binding to distinguish between Zn(II) and Co(II). Neither metal ion is released from the protein until the acid conformational change begins. In addition, Hg(II), expected in model complexes to form the most stable complex with a great variety of donor atoms, does not stabilize the protein against the

conformational change below pH 4.0. A similar change occurs above pH 11.0 (Figure 2), which may account for the loss of inhibitor binding observed in this pH range (Lindskog, 1963). Urea induces similar rotatory changes and has been shown to result in the release of the zinc ion (Lindskog and Malmström, 1963).

Binding of acetazolamide to carbonic anhydrase does not detectably alter the general or aromatic structure of the protein as reflected in the ultraviolet ORD. Likewise the thiadiazole ring does not undergo any detectable change in environment on binding to the protein, since its absorption band shows no spectral shift and does not become optically active. However, sulfonamide binding does markedly alter the molecular structure immediately surrounding the metal ion, as shown by the shifts in the visible spectra of the cobalt enzyme and in the optical activity associated with these bands.

The uninhibited cobalt enzyme shows at least four visible absorption bands representing the d–d electronic transitions of the cobalt ion, separated by 1100, 1800, and 700 cm^{-1} (Figure 6). Such band splitting could arise either from splitting of the d orbital energy levels induced by a square-planar distortion (Dunn, 1960) or by the spin-orbital coupling observed in some regular tetrahedral complexes of Co(II) (Cotton and Wilkinson, 1962). Splitting in the latter circumstance has been shown to be several hundred cm^{-1}, considerably less than that observed in the cobalt carbonic anhydrase bands (Dunn, 1960). Lack of optical activity in any of these bands supports a square-planar distortion as the cause of the observed splitting (Figure 4 and 5).

Binding of acetazolamide or cyanide to the cobalt enzyme causes a red shift in the major transitions, intensifies the maxima, and reduces but does not abolish the band splitting. Most significantly both agents induce optical activity in the cobalt bands. The rather marked asymmetry of the positive Cotton effects induced indicates that at least two transitions are optically active and implies that the complex does not have a regular geometry. Square-planar complexes of both Co(II) and Co(III) have a marked tendency to add perpendicular ligands, converting the complex to an octahedron or distorted octahedron and displacing distantly coordinated water molecules if they are present (Falk and Phillips, 1964). Such additions are accompanied by red shifts in the absorption maxima (Kon, 1955; Falk and Phillips, 1964), as observed for both the acetazolamide- and CN^--inhibited cobalt enzymes. Addition of a perpendicular ligand would destroy the plane of symmetry and thus make the complex optically active.

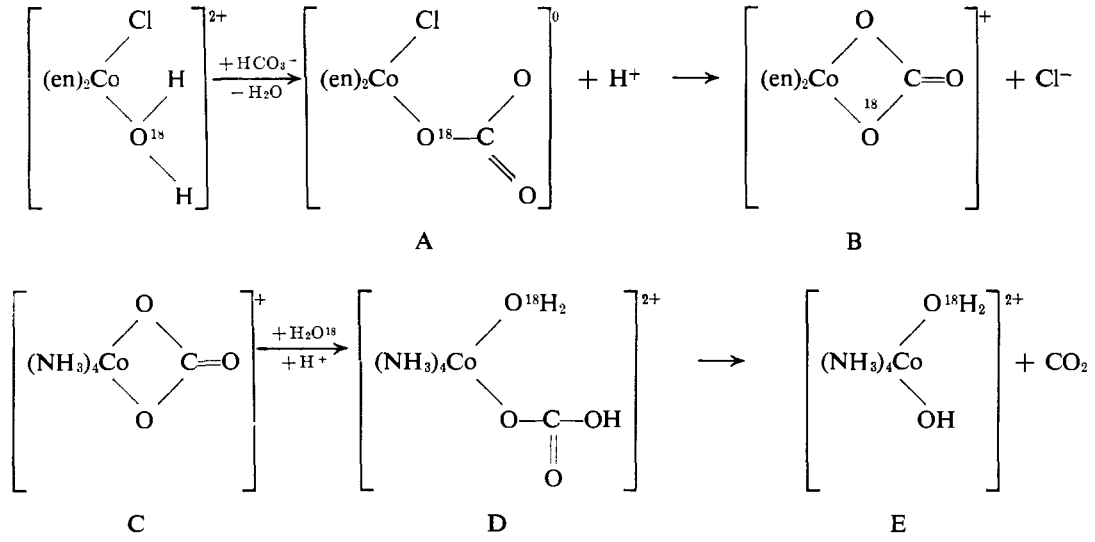
A square-planar configuration at the active site of cobalt carbonic anhydrase, as well as being compatible with the ORD data, would explain the high sensitivity of the cobalt enzyme to cyanide, 2.2×10^{-5} M being sufficient for 50% inhibition, and the rather marked preference for the mixed enzyme–cobalt–cyanide complex over the cobalt–cyanide complex, 50–100-fold molar excess cyanide being required before cobalt is removed (Figure 5). It would also be compatible with the observation that bidentate chelating agents are very poor inhibitors of the enzyme as compared to a variety of monodentate coordinating ions (Davis, 1961; Lindskog, 1963). Binding of acetazolamide to carbonic anhydrase is known to require the presence of a metal ion, cobalt or zinc (Lindskog, 1963). If the sulfonamide coordinates the metal ion and has a protein-binding site as well, an additional chelate ring would be formed which could induce marked dissymmetry, not incompatible with the large Cotton effects generated by acetazolamide binding.

Though inhibitor binding to the active site of carbonic anhydrase involves the catalytically essential metal ion, the binding of inhibitors may or may not bear any direct relationship to the mode of CO_2 or HCO_3^- binding, assuming the latter to be the substrate in the dehydration reaction (DeVoe and Kistiakowsky, 1961). Kinetic studies have indicated a noncompetitive type of inhibition for the sulfonamide and metal-binding inhibitors when employed in the hydration reaction (Davis, 1961). This has led to the hypothesis that substrate binding occurs at some site other than the metal ion (Davis, 1961).

Addition of HCO_3^- to the cobalt enzyme induces immediate and rather marked changes in the spectrum (Figure 6A). Thus bicarbonate is able to react with the cobalt enzyme in a manner that markedly affects the energies of the d orbitals surrounding the metal ion. However, the geometry of the complex as reflected in the ORD shows only a minor, but detectable shift (Figure 7). Since treatment of the acetazolamide-inhibited cobalt enzyme with 0.5 M HCO_3^- causes no change in the spectrum or in the Cotton effects induced by acetazolamide (Figure 4), the reaction of HCO_3^- ion would appear to be at the cobalt site, probably within its coordination sphere. The reaction kinetics at the highest concentration of HCO_3^- employed here have not been studied; however, kinetic studies of the dehydration reaction (DeVoe and Kistiakowsky, 1961; Gibbons and Edsall, 1964) have employed HCO_3^- concentrations as high as 0.1 M and no deviations from Michaelis–Menten kinetics were observed.

The hypothesis that the development of this spectral change represents the accumulation of an enzyme–substrate intermediate is supported by a plot of $-\Delta\epsilon$ at 640 mμ as a function of HCO_3^- concentration which generates a hyperbola. The spectral change is half-complete at 8×10^{-2} M HCO_3^-, similar in magnitude to the Michaelis constant, 3×10^{-2} M, reported for human zinc carbonic anhydrase B catalyzing the dehydration reaction (Gibbons and Edsall, 1964). At the enzyme concentration employed for the spectral studies, equilibrium would be established between the forward and back reactions almost immediately. At pH 8.2, the equilibrium is 60-fold in favor of HCO_3^-, hence there would be only slow change in the concentration due to the gradual evolution of CO_2. Bicarbonate induces no detectable changes in the ultraviolet ORD curves of either the zinc or cobalt enzymes and hence would not appear to induce a major change in protein structure.

SCHEME I



Carbonato complexes of metal ions have been known since the earliest studies of coordination compounds (Werner and Vilmos, 1899). Of particular interest here are the studies involving mixed carbonato complexes of cobalt with ethylenediamine and ammonia. Optically pure D-$[Co(en_2)Cl \cdot H_2O]^{2+}$ can be converted to optically pure D-$[Co(en_2)CO_3]^+$ by reaction with sodium bicarbonate. Oxygen-18 has been used to elucidate the mechanism as pictured below. Bicarbonate adds to a coordinated water molecule, losing one of its original oxygen atoms, forming an intermediate, probably A (see Scheme I) (Hunt *et al.*, 1952; Stranks and Harris, 1952; Posey and Taube, 1953; Sargeson, 1964). Under the conditions of reaction, the intermediate goes on to form the highly distorted carbonato complex B (Barclay and Hoskins, 1962). The diaquo ion can be regenerated with full retention of optical activity by dissolving the carbonato complex in acid solution. Oxygen-18 studies with the corresponding tetraamine have indicated the reaction to involve C, D, and E (Posey and Taube, 1953). Maintenance of the Co–O bond explains the observed retention of optical activity. With the proper electronic structure of the cobalt ion and in the presence of adjacent functional protein groups, perhaps to protonate and deprotonate the intermediate, such exchanges might be extremely rapid and could catalyze the release of the CO_2 molecule. The possible enzymatic advantage of such an intermediate would be that, while the oxygen of the coordinated water molecule or OH^- preserves its nucleophilic character as indicated by the ^{18}O exchange data, a coordinated oxygen would also become a better leaving group in the dehydration reaction. The breaking of the C–O bond in this mechanism is not unlike the hydrolytic cleavage observed in a variety of esters, as has been pointed out by Posey and Taube (1953), and it is of interest that carbonic anhydrase has esterase activity, also limited to the zinc and cobalt enzymes (Tashian *et al.*, 1963; Malmström *et al.*, 1964).

The spectral changes observed in cobalt carbonic anhydrase on the addition of HCO_3^- (Figure 6) are very similar to those reported by Lindskog and Nyman (1964) when the enzyme is exposed to increasing H^+ concentrations. The pH-dependent spectral change occurs without loss of cobalt (Figure 2) and like the change occurring on the addition of HCO_3^- is accompanied by a small but reproducible perturbation in the ORD (Figure 7). Loss of absorption at 640 mμ with decreasing pH is described by a curve with an inflection point at pH 7.5 and was interpreted to reflect the uncoupling of one of the protein ligand groups at low pH (Lindskog, 1963; Lindskog and Nyman, 1964). The protonation of a coordinated OH^- ion could not be ruled out.

A change in the nature of a monodentate ligand coordinated to the cobalt would be less damaging to the original geometry than the breaking of a chelate ring, a necessity if a protein donor atom were involved in these changes. Assuming a square-planar complex with a distantly coordinated water molecule to be the configuration of cobalt carbonic anhydrase at pH 5.5, this would become an OH^- ion by pH 9.0 and would be replaced by the addition of bicarbonate. The participation of an enzyme-bound OH^- ion in the hydration reaction as has been postulated (DeVoe and Kistiakowsky, 1961) would be readily accomplished. The various monodentate ligands involved fall in the spectrochemical series as follows: $^-OH < CO_3^{2-} \leq H_2O \ll CN^-$. Hence, OH^- which produces the smallest ligand field would be expected to create the least perturbation of a nonoptically active geometry, CO_3^{2-} or HCO_3^-, and H_2O would produce somewhat more, while cyanide at the other end of the series would create a major perturbation, predictions compatible with the ORD data (Figures 5 and 7).

The existence of a square-planar configuration at the

metal-binding site of carbonic anhydrase is also supported by the spectral and ORD data on the copper enzyme (Figure 8). Among the transition metal ions, Cu(II) has the greatest tendency to induce the square-planar distortion, and the majority of copper complexes are either square planar or distorted octahedra with two distant *trans* ligands. The spectrum of copper carbonic anhydrase has a profile not unlike the spectra of square-planar copper complexes, with two and possibly three transitions represented by the broad asymmetric absorption band (Cotton and Wilkinson, 1962; Dunn, 1960). There is no significant optical activity in the region of the main copper absorption band. Although copper carbonic anhydrase is enzymatically inactive in the hydration of CO_2 (Table I), cyanide combines with the copper enzyme with moderate intensification of the spectrum and shift of the maximum from 750 to 700 mμ. Most significantly the copper absorption bands become optically active on complexation with CN^-. Thus the complex lacks a plane of symmetry. In view of the known chemistry of copper, such an alteration would seem to be most probably toward a distorted octahedron by the close approach of a fifth ligand rather than distortion toward a tetrahedral geometry.

Addition of perpendicular ligands to square-planar complexes of Cu(II) is more difficult than to corresponding cobalt complexes. A 10-fold molar excess of CN^- is required to fully develop the spectral and ORD changes of copper carbonic anhydrase while only a 1:1 molar ratio is required in the case of the cobalt enzyme. Cobalt appears to be the only transition metal capable of serving a catalytic function of any magnitude at the active site of carbonic anhydrase (Table I) (Lindskog and Nyman, 1964; Malmström *et al.*, 1964). This could be related to the readily reversible addition of monodentate ligands observed in a variety of cobalt complexes and the reluctance of other first transition metal complexes to undergo similar exchanges.

The identity of the donor atoms at the active site or the amino acid residues contributing them is unknown. Prevention of ^{65}Zn(II) binding to the apoenzyme by sulfhydryl reagents seemed to indicate the involvement of the single sulfhydryl group of the human enzyme in zinc binding (Rickli and Edsall, 1962). However, recent X-ray diffraction studies (Malmström *et al.*, 1964) would seem to indicate that it is located some distance from the zinc-binding site. The "buried" nature of the histidine residues has suggested the possible function of at least one imidazole nitrogen as a donor atom (Riddiford, 1965). An equally valid argument could be made for the "buried" tyrosines. The series of stability constants determined for the first transition and II-B metallocarbonic anhydrases at pH 5.5, by partially removing the metal ions with chelating agents (Lindskog and Nyman, 1964), is difficult to interpret because of the conformational changes occurring in the apoenzyme at this pH. While the identification of the donor atoms must await further study, the present work indicates some of the conformational features of the metal-binding site.

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Stereochemical Aspects of the Substrate Specificity of Horse Liver Alcohol Dehydrogenase*

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ABSTRACT: The steric course of reduction of a number of 3- and 4-alkylcyclohexanones and the four 10-methyl-2-decalones has been determined. Marked rate differences between the enantiomers in both the *cis*- and *trans*-decalone series allowed resolution of the *dl* pairs. The reduction of (−)-3-methylcyclohexanone, *l*-10-methyl-*trans*-2-decalone, *d*-10-methyl-*cis*-2-decalone, and *l*-10-methyl-*cis*-2-decalone proceeded stereospecifically while the other substrates gave mixtures of axial and equatorial alcohols. From a correlation of observed rates and steric specificity, areas of a substrate that are consistent with high enzymatic activity, and inhibition areas, have been defined. Quantitative inhibition factors, allowing prediction of reaction rates and the stereochemistry of reduction, have been derived in a number of instances and a mapping of the steric relationship of enzyme, coenzyme, and substrate in the neighborhood of the active site has been proposed.

A number of investigators have demonstrated that crystalline horse liver alcohol dehydrogenase (liver ADH)[1] efficiently catalyzes the nicotinamide–adenine dinucleotide (NAD)-dependent ketone–alcohol interconversion of many derivatives of cyclohexanone and cyclohexanol (Winer, 1958; Merrit and Tomkins, 1959; Prelog, 1963, 1964). Studies (Prelog, 1963, 1964) of the oxidation and reduction rates of 2-methylcyclohexanones, 2-methylcyclohexanols, and certain 1-decalones and 1-decalols established a 2-alkyl inhibitory effect and led to proposals concerning the relative location of the coenzyme and substrate in the ternary complex; the presumed importance of the nonbonded interactions of the substrate with the carboxamide group of the coenzyme was stressed as an important factor in the determination of substrate specificity.

In the present paper, the hitherto unexplored reduction path of 3- and 4-alkylcyclohexanones and of the four 10-methyl-2-decalones is reported. From a correlation of observed rates and steric specificity it has been found possible to define areas of a substrate that are consistent with high enzymatic activity and inhibition areas that lead to rate decreases. In a number of instances, quantitative inhibition factors that allow the prediction of reaction rates have been derived. From these results and from a number of examples that ap-

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[1] Abbreviations used are: liver ADH, horse liver alcohol dehydrogenase; NAD, nicotinamide–adenine dinucleotide; $NADH_2$, reduced NAD; nmr, nuclear magnetic resonance; vpc, vapor phase chromatography; ORD, optical rotatory dispersion.