Extrinsic Cotton Effects in a Metal Chelator–Bovine Carbonic Anhydrase Complex*

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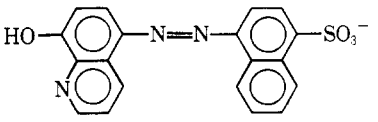
ABSTRACT: The esterase activity (*p*-nitrophenyl acetate as substrate) of bovine carbonic anhydrase B is completely inhibited by the binding of 1 molecule of the metal-chelating agent 4-(8-hydroxy-5-quinolylazo)-1-naphthalenesulfonate (I) to the enzyme, with an inhibitor constant of 5.0×10^{-4} M at pH 8, 25°. The binding is accompanied by a change in the visible absorption of I. The fact that the kinetics of this spectral change at pH 8 is too fast for observation in a stopped-flow apparatus sets an approximate lower limit of 5×10^{6} M^{-1} for the apparent second-order rate constant for the interaction of I with the enzyme. The binding of I to the enzyme induces asymmetry into electronic transitions contributing to its visible absorption, with resulting extrinsic optical rotatory dispersion (ORD). Quantitative observations of the ORD show that the zinc atom at the active site of the enzyme is essential for its appearance; that a single molecule of I is bound in the vicinity of the active site to produce the extrinsic ORD; and that the effect is abolished by addition of a single molecule of a more tightly bound sulfonamide inhibitor. In the presence of I additional Zn^{2+} ions can be bound to the enzyme, with pronounced alterations in the overall extrinsic ORD. These alterations are reversed by addition of EDTA, either by removal of the excess Zn^{2+} or by displacement of I from the enzyme–zinc–chelator complex.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a metalloenzyme containing one atom of very tightly bound zinc(II) per molecule of 30,000 molecular weight. Lindskog and Malmström (1962) and Lindskog (1963) have shown that the zinc atom can be removed at low pH in the presence of metal chelators such as 1,10-phenanthroline, with complete loss by the enzyme of its catalytic properties, and that fully active enzyme, apparently native in all respects, can be regenerated by the addition of 1 equivalent of Zn^{2+}.

We have investigated the binding of a metal-chelating agent to carbonic anhydrase, selecting for this purpose a substance having optical absorption in the visible region of the spectrum and a solubilizing group, 4-(8-hydroxy-5-quinolylazo)-1-naphthalenesulfonate (I). Experiments with this material indicate that the zinc in the native enzyme is required for binding to take place, as judged by optical means; that the enzyme with chelator bound is enzymically inactive, and that the chelator is bound in an asymmetric environment. Evidence is obtained for the binding of additional zinc ions to the enzyme in the presence of the chelating agent.



I

Materials and Methods

Bovine carbonic anhydrase B was prepared, from commercial samples obtained from Worthington Biochemical Corp., Freehold, N. J., by chromatography on DEAE-cellulose according to the method of Lindskog (1960). Atomic absorption measurements with a Perkin-Elmer Model 303 spectrophotometer showed that the preparations contained 1.00 ± 0.05 atom of zinc per molecule, assuming an absorptivity at 280 nm of 5.7×10^{4} M^{-1} cm^{-1} for the enzyme (Lindskog and Nyman, 1964). Enzymic activities were determined with *p*-nitrophenyl acetate as substrate using the method previously described (Henkens and Sturtevant, 1968). Specific activities of 0.31–0.34 sec^{-1} were observed for the various enzyme preparations used.

The zinc-free apoenzyme was prepared by prolonged dialysis against 1,10-phenanthroline in 0.1 M acetate buffer at pH 5.2 and 5° (Lindskog and Malmström, 1962).

4-(8-Hydroxy-5-quinolylazo)-1-naphthalenesulfonic acid, I, was purchased from Eastman Organic Chemicals, Rochester, N. Y. It was recrystallized as the sodium salt from 0.1 N NaOH, washed on the filter with cold water followed by cold ethanol and dried *in vacuo*. Stock solutions were prepared fresh on the day of use.

Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide, Diamox) was obtained from American Cyanamide Co., Pearl River, N. Y. It was recrystallized three times from water. Stock solutions were prepared in dilute NaOH.

Absorbance measurements were made with a Cary 14 spectrophotometer. Optical rotatory dispersion (ORD) was measured with a Cary 60 spectropolarimeter. The cells used were of cylindrical form made of fused silica of low birefringence, having either 2- or 10-mm optical path length. The slit width was generally 2 mm, corresponding to a spectral bandwidth of 16 nm at a wavelength of 500 nm. Difference ORD was measured using the differential cell holder supplied by the manufacturer. We observed rotatory artifacts using this holder of the type previously reported by Adkins and Yang (1968). However these artifacts were circumvented in our

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work by always placing the essentially nonabsorbing enzyme solution in the reflected beam.

Results and Discussion

Visible Difference Spectrum. Binding of I to bovine carbonic anhydrase B causes a blue shift and a decrease in bandwidth of the absorption spectrum of I in the region 400–600 nm. The visible spectrum of I (curve B) and of its complex with carbonic anhydrase (curve A), observed at 25° and pH 7.9 in 0.008 M Tris-HCl buffer, are shown in Figure 1. The spectrum of the complex is calculated from the measured difference spectrum of I plus enzyme *vs.* I, and the formation constant for the complex which, as shown later, was determined independently from two phenomena, enzyme inhibition and difference polarimetry. At dye concentrations greater than 2×10^{-4} M there are slight shifts in the positions of the peak, troughs, and isosbestic points.

No difference spectrum is observed in the visible region of the spectrum when I is added to apocarbonic anhydrase. The difference spectrum appears, however, on addition of Zn^{2+} to form the reconstituted enzyme. These observations suggest either that no binding of the inhibitor takes place in the absence of Zn(II) or that if it does take place it is relatively weak since it does not detectably perturb the spectrum of the inhibitor.

The rate of appearance of the difference spectrum on addition of chelating agent to the active enzyme was too rapid to be measured with 1.5×10^{-4} M I and 3.8×10^{-5} M carbonic anhydrase, in a stopped-flow spectrophotometer with an instrumental dead time of less than 3 msec (Sturtevant, 1964). This places a lower limit of approximately 5×10^{6} M^{-1} sec^{-1} on the apparent rate constant for the reaction. Taylor *et al.* (1970a,b) have observed rate constants for the reactions at 25° and pH 6.5, where the rates are approximately one-third what they are at pH 7.9, of a wide variety of sulfonamide inhibitors with human carbonic anhydrase C in the range 5×10^{4} to 10^{7} M^{-1} sec^{-1}. Rudolph (1970) observed a rate constant for the reaction of 5-dimethylaminonaphthalene-1-sulfonamide with the bovine enzyme of 2.7×10^{5} M^{-1} sec^{-1} at 25°, pH 6.5, and Taylor *et al.* (1970a) found 2.4×10^{5} M^{-1} sec^{-1} for this same inhibitor with the human enzyme. It thus appears that the two enzymes have similar reaction rates with inhibitors, and that the reaction of I with the bovine enzyme has a rate lying within the range to be expected for the binding of sulfonamide inhibitors to this enzyme.

Inhibition of Esterase Activity. The carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate is reversibly inhibited by I. The fractional inhibition caused by a given concentration of I was found to remain constant with a 25-fold variation of substrate concentration. Although this observation does not prove that the inhibition is noncompetitive, since it probably simply reflects weak substrate binding, it nevertheless permits ready determination of the inhibitor constant, K_I. For either noncompetitive inhibition, or competitive inhibition with weak substrate binding, the relative activity, θ, in a system following Michaelis–Menten kinetics is given by the relation

$$\theta = \frac{r}{r_0} = \frac{K_I}{K_I + (I)} \qquad (1)$$

where r and r_0 are the initial rates in the presence and absence of inhibitor, respectively, (I) is the concentration of unbound inhibitor, usually practically equal to the total concentration of inhibitor, $(I)_0$, and K_I is the dissociation constant of the enzyme–inhibitor complex, and also, in the case of noncompetitive inhibition, of the enzyme–substrate–inhibitor ternary complex. A plot of θ as a function of $(I)_0$ is given in Figure 2. Since

$$\log \frac{\theta}{1 - \theta} = \log K_I - \log (I)_0 \qquad (2)$$

K_I can be evaluated from the intercept of a plot of $\log [\theta/(1 - \theta)]$ *vs.* $\log (I)_0$, which of course should have a slope of -1. The insert in Figure 2 is a plot of this type, and leads to a value of 5.0×10^{-4} M for K_I at pH 8.0 in a 0.01 M Tris-HCl buffer.

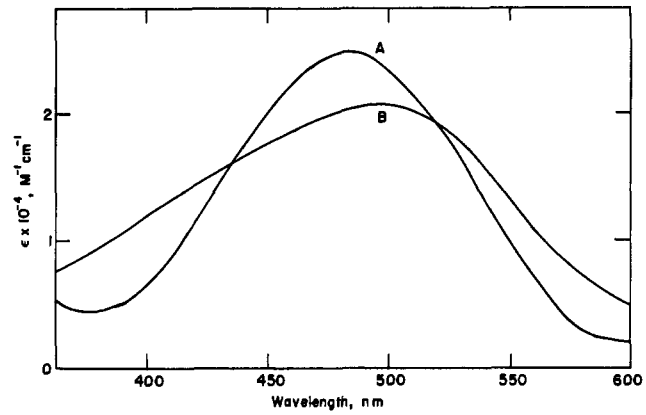
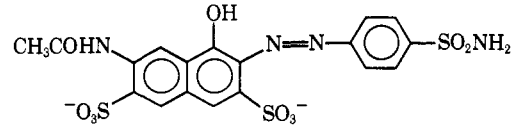


FIGURE 1: Visible absorption spectrum of I at pH 7.9 in 0.008 M Tris·HCl buffer (curve B). Visible absorption spectrum of the complex of I with bovine carbonic anhydrase B under the same conditions (curve A). The latter spectrum was calculated from the measured difference spectrum of I plus enzyme *vs.* I alone, with correction for incompleteness of the complex formation.

The inhibitory action of I would suggest that it is bound in much the same way as sulfonamide inhibitors, the binding of which is thought to involve hydrophobic interactions plus coordination to the zinc atom of the anionic form of the sulfonamide group (Chen and Kernohan, 1967; Coleman, 1967a, 1968; Taylor *et al.*, 1970b). It is interesting that I is much more loosely bound to the enzyme than are many sulfonamides in spite of being at least as rapidly bound. It is evident that the rate of dissociation of I must be much higher than that of most of the sulfonamides. For example, the off rate for 5-dimethylaminonaphthalene-1-sulfonamide at pH 8.1 is 0.17 sec^{-1} (Rudolph, 1970). From the lower limit for the on rate for I given above, and its dissociation constant, we can conclude that the off rate for I is at least 2500 sec^{-1}. This is three orders of magnitude larger than the off rate for any sulfonamide inhibitors which have been investigated (Taylor *et al.*, 1970a).

Induced Optical Activity. Binding of optically inactive I to carbonic anhydrase results in induction of asymmetry in the transitions responsible for the absorption bands of I in the vicinity of 500 nm. Similar cases of extrinsic Cotton effects resulting from the binding of 2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate, II, to sev-



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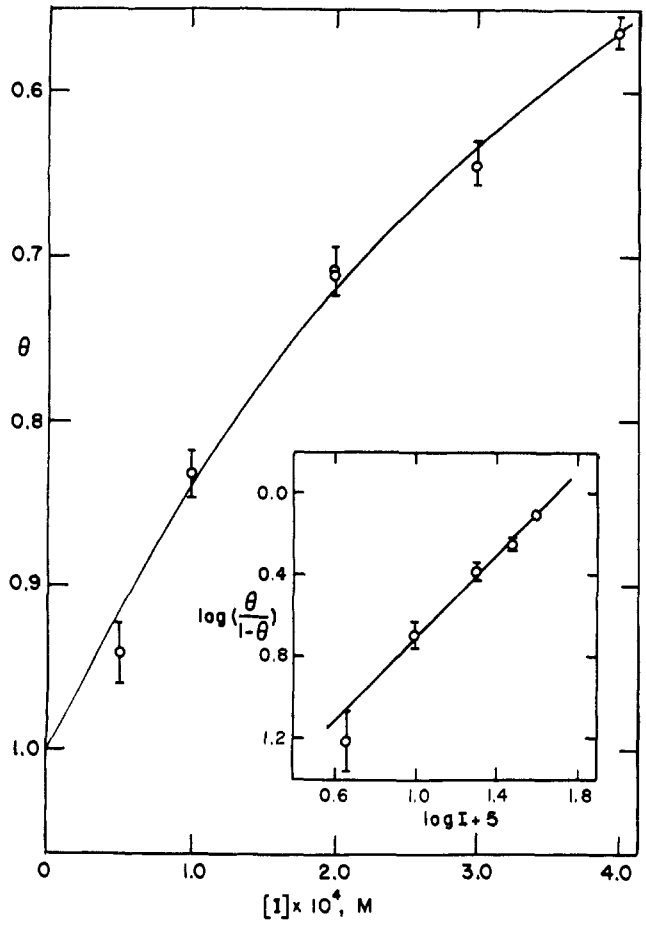

FIGURE 2: Inhibition of the esterase activity of carbonic anhydrase by I in 0.01 M Tris·HCl buffer, pH 8, 25°. Substrate, 2.4 × 10^{-4} M *p*-nitrophenyl acetate. Relative activity, θ, is plotted against concentration of I. Insert is a plot of log $[\theta/(1-\theta)]$ *vs.* log $(I)_0$, with the theoretical slope of −1.

eral different carbonic anhydrases have been extensively studied by Coleman (1967b, 1968). In Figure 3 the optical rotatory dispersion of carbonic anhydrase in the region 300–600 nm (curve B) is compared with that of the enzyme plus I (curve A). Curve B shows an extrinsic Cotton effect (or effects) due to the binding of I superimposed on the plain dispersion of the enzyme. The fact that the ORD on either side of the extrinsic Cotton effect(s) is completely unaltered shows that the binding of I has no significant effect on the optically active absorption bands of the enzyme, and thus presumably produces no more than minor local changes in the conformation of

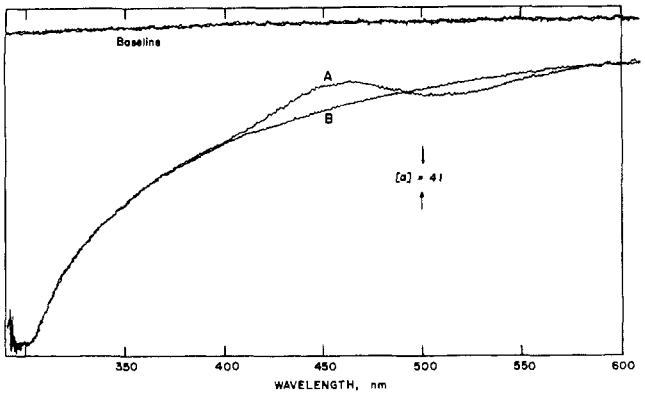

FIGURE 3: Curve B, ORD of 8.0 × 10^{-5} M carbonic anhydrase in 0.008 M Tris·HCl buffer (pH 7.9). Curve A, ORD of 8.0 × 10^{-5} M carbonic anhydrase plus 6.7 × 10^{-5} M I under same conditions.

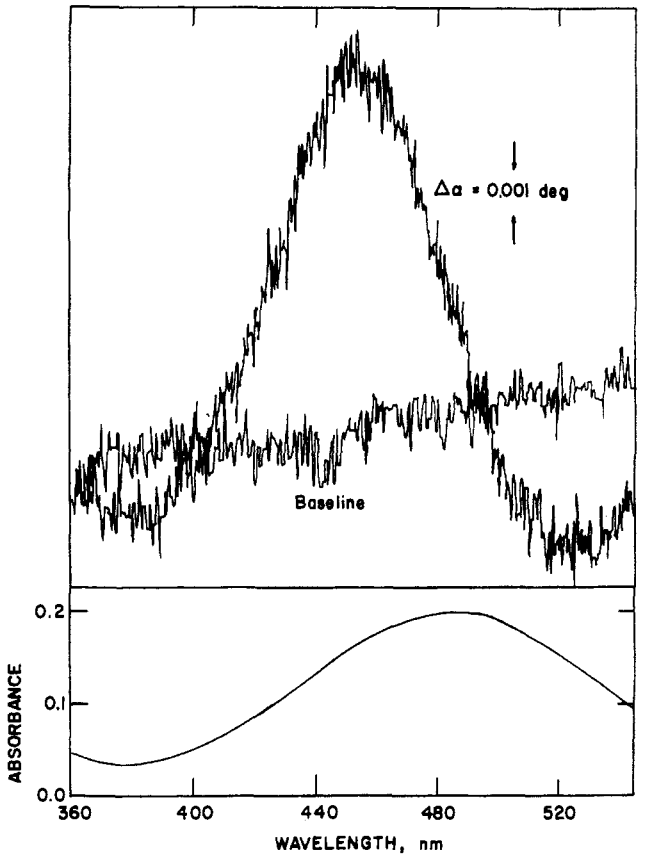

FIGURE 4: Difference ORD; sample cell contained same solution as for curve A of Figure 4, and reference cell contained same solution as for curve B. The visible absorption spectrum of the enzyme–I complex is shown in the lower part of the figure. Optical path length 10 mm.

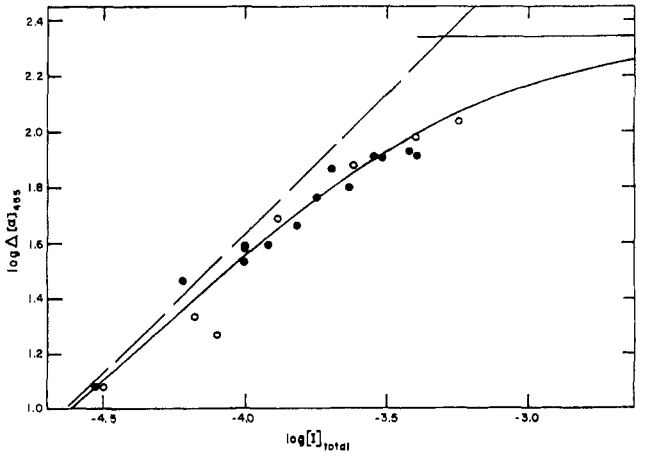

FIGURE 5: Difference in specific rotation at 455 nm between the carbonic anhydrase–I complex and the enzyme alone, plotted as a function of the total concentration of I, 0.05 M Tris·HCl buffer (pH 8.0). Open circles, sample and reference cells both made up with native enzyme; filled circles, sample cell made up with zinc-reactivated apoenzyme, reference cell with apoenzyme; 5 × 10^{-4} M EDTA in both cells. The solid curve is calculated using eq 2, with $\theta = \Delta[\alpha]/\Delta[\alpha]_\infty$, $K_I = 5 \times 10^{-4}$ M, where $\Delta[\alpha]_\infty$, the change in specific rotation at saturation, is equal to 2.2 × 10^2.

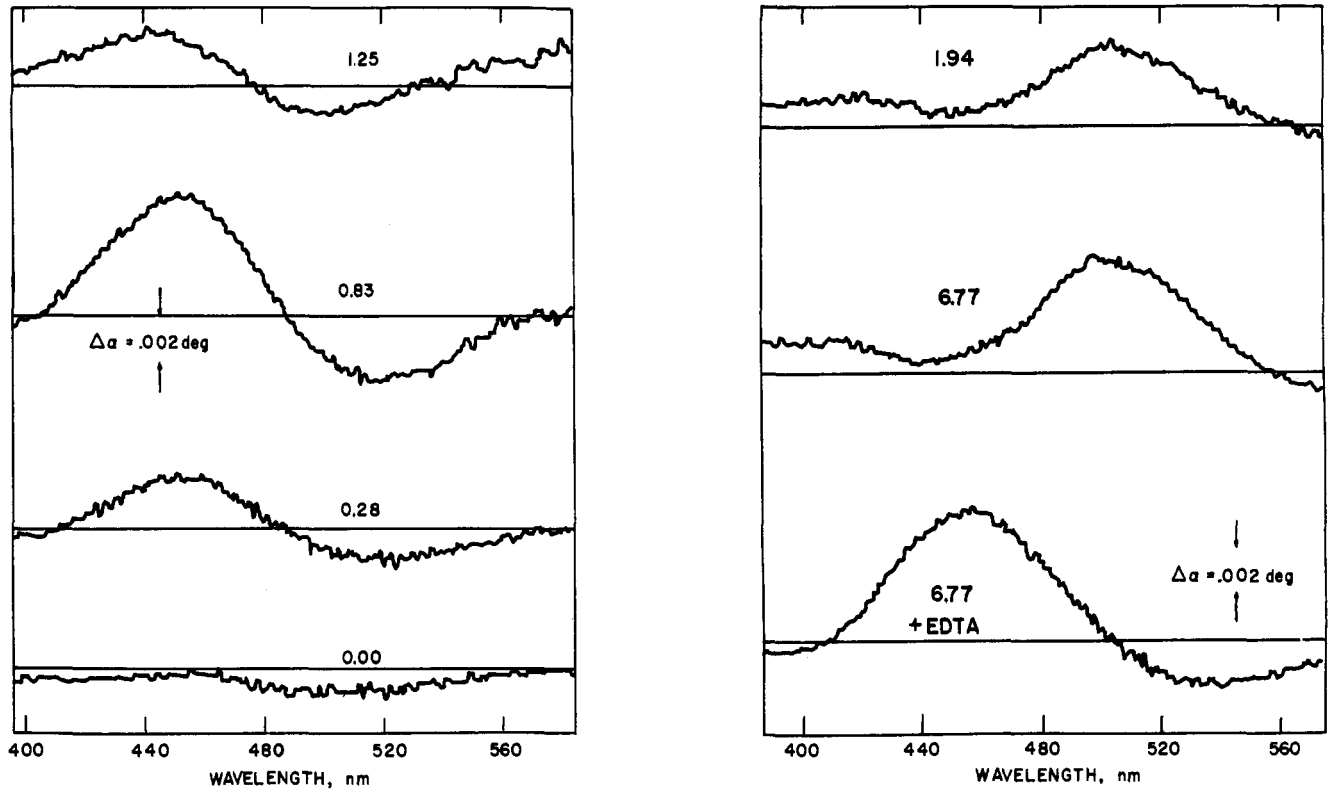

FIGURE 6: ORD difference curves illustrating changes occurring on titration of a solution of apocarbonic anhydrase containing excess I with Zn^{2+}. The sample cell contained 4.8×10^{-5} M protein and 1.0×10^{-4} M I in 0.01 M Tris·HCl buffer (pH 8.0) and the reference cell the same components except for omission of I. The figures next to the curves give the moles of Zn^{2+} added per mole of enzyme. For the bottom curve on the right side, 5×10^{-4} M EDTA was added to both the sample and reference cells. Path length 10 mm.

the protein. The difference ORD of the I–carbonic anhydrase complex relative to carbonic anhydrase is shown in the upper part of Figure 4. The lower part of Figure 4 gives the absorption spectrum of the I–enzyme complex for purposes of comparison.

The Cotton effect molar amplitude, defined as the difference between the molecular rotation at a peak or trough and the molecular rotation of the next extremum at shorter wavelength divided by 100, calculated from Figure 4 is $-1200°\ M^{-1}$, after making allowance for incomplete formation of the complex under the experimental conditions. Coleman (1967b, 1968) observed a molar amplitude of $+1200°\ M^{-1}$ in the binding of II to human carbonic anhydrase.

The difference specific rotations at 455 nm observed in the titration of carbonic anhydrase with I are shown in Figure 5 as a function of the total concentration of I. The solid line was calculated using the dissociation constant, 5.0×10^{-4} M, deduced from the enzyme inhibition experiments. It is clear that the full optical effect is produced by the binding of 1 molecule of I per molecule of enzyme, as is also true of the enzyme inhibition.

Role of Zn(II). As was found to be the case in connection with the optical difference spectrum, no extrinsic Cotton effects are observed when I is added to the apoenzyme. Addition of Zn^{2+} to a solution of apoenzyme containing excess I results in the appearance of the same Cotton effects as observed with the native protein plus I, with the amplitude proportional, as expected, to the amount of Zn^{2+} added up to one ion per molecule of protein. Further addition of Zn^{2+}, however, leads to unexpected results, as shown in Figure 6, in which the difference ORD is given for various ratios of Zn^{2+} to the apoenzyme. As excess Zn^{2+} is added, both the negative peak near 520 nm and the positive peak near 450 nm decrease in amplitude; when the excess of Zn^{2+} exceeds about 40%, the negative peak actually changes sign and finally obtains a positive amplitude considerably larger than the original negative amplitude and shifted somewhat toward the blue. Addition of EDTA completely removes the effects produced by excess Zn^{2+}, although, as shown in Figure 6, the position of the negative peak is for some unknown reason shifted slightly toward the red from that of the original peak.

EDTA does not remove Zn(II) from the active-site crevice (Fridborg *et al.*, 1967) of carbonic anhydrase at neutral pH, because of the extremely low rate of dissociation of the zinc (Henkens and Sturtevant, 1968). Its abolishment of the positive Cotton effect produced by excess Zn^{2+} in the presence of I can either result from displacing molecules of I involved in enzyme–zinc–I ternary complexes, or from simple sequestering of the excess bound zinc. The former possibility seems less likely than the latter since EDTA is obviously unable to displace I from the enzyme–active-site zinc–I ternary complex responsible for the negative Cotton effect.

The above results provide evidence for the binding by carbonic anhydrase of zinc ions in addition to the active-site zinc in the presence of I. The stoichiometry of this additional binding, and whether any Zn^{2+} is bound in the absence of a third component such as I, are unanswered questions.

Fabry *et al.* (1970) have reported that both Zn^{2+}– and Co^{2+}–carbonic anhydrase (bovine isoenzyme B) bind one additional Co^{2+} ion which is not removed by prolonged dialysis. The extra Co^{2+} is presumably not bound near the active site since the activity of the enzyme is not affected by its presence. The fact that enzyme regenerated from the apoenzyme by the addition of excess Zn^{2+} is found after dialysis to con-

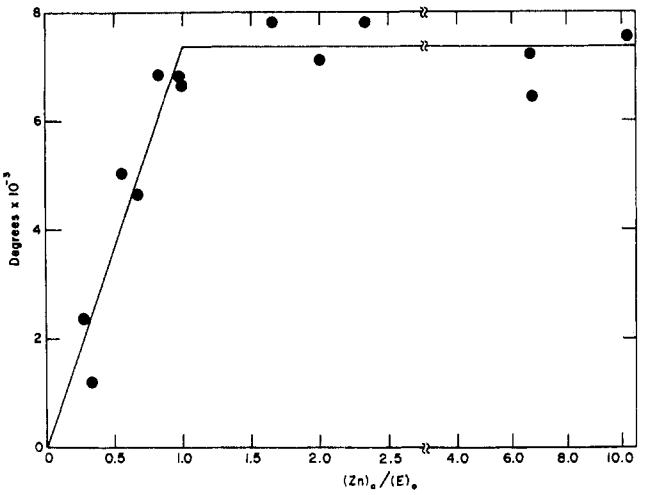

FIGURE 7: Difference between the rotations at the peak and the trough of the extrinsic Cotton effect centered at 500 nm as a function of the ratio of added Zn^{2+} concentration to enzyme concentration. The sample cell contained 4.8×10^{-5} M apocarbonic anhydrase, 1.0×10^{-4} M I, and the indicated concentrtion of Zn^{2+} in 0.01 M Tris·HCl buffer (pH 8.0); the reference cell contained the same solution with I omitted. To avoid nonspecific binding of Zn^{2+} (see text), EDTA was added to each solution after the addition of Zn^{2+}.

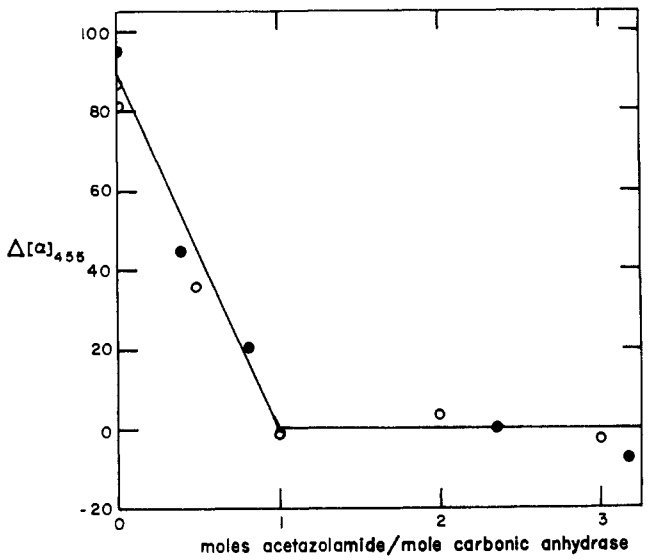

FIGURE 8: Effect of the addition of acetazolamide to a solution of the carbonic anhydrase–I complex on the relative specific rotation, $\Delta[\alpha]_{455}$, of the extrinsic Cotton effect peak at 455 nm. 1.0×10^{-4} M enzyme and 4.0×10^{-4} M I in 0.05 M Tris·HCl buffer (pH 8.0) open circles, apoenzyme reactivated by Zn(II), 5×10^{-4} M EDTA added; filled circles, native enzyme, no EDTA added.

tain only one atom of zinc per molecule shows that no such tight binding as reported for Co^{2+} by Fabry *et al.*, takes place with Zn^{2+}. It is evident that the additional binding of Zn^{2+} observed in the presence of I is of an entirely different character since more than two Zn^{2+} per mole of apoenzyme are required to produce the maximum positive Cotton effect (Figure 6).

The stoichiometric requirement for Zn^{2+} is shown in Figure 7, which gives an ORD titration curve of the apoenzyme plus chelator with Zn^{2+}. Interference by nonspecific binding was avoided in these experiments by the addition of EDTA after each addition of Zn^{2+}.

Many sulfonamide inhibitors of carbonic anhydrase are bound extremely tightly and would thus be expected to displace I from its complex with the enzyme. Figure 8 gives a titration curve which shows that addition of 1 mole of acetazolamide ($K_{diss} \approx 10^{-8}$ M) per mole of enzyme causes complete removal of the Cotton effects due to the binding of I. This observations gives further support to the view that the extrinsic ORD produced by I results from binding of a single molecule of I in the immediate vicinity of the active site.

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