Unique Conformational Properties of Muscle Carbonic Anhydrase III as Demonstrated by Circular Dichroism Spectrometry†

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ABSTRACT: Circular dichroic spectra of rabbit muscle carbonic anhydrase III have been obtained and compared with those of rabbit erythrocyte carbonic anhydrases I and II. In particular, the effects of pH, relative zinc content, the sulfhydryl reducing agent dithiothreitol, and the highly specific carbonic anhydrase inhibitor acetazolamide on these spectra were studied. The muscle isoenzyme III was found to display elliptical bands which are similar to those of the type I and II isoenzymes with respect to sign and λ_{max} values, but which vary in intensity as compared with the erythrocyte isoenzymes and with values reported in the literature for carbonic anhydrases I and II. As an unusual feature of the carbonic anhydrase III spectrum, very intense positive bands in the far ultraviolet at 222 nm ($[\theta]_M = 4.87 \times 10^5$) and at 232 nm ($[\theta]_M = 1.13 \times 10^5$) were observed which were interpreted to reflect altered conformational states of this protein. Carbonic anhydrase III in the holoenzyme form was found to be stable in the pH range 5–11, but in its apoenzyme form (in contrast to the results reported in the literature for isoenzymes I and II) it underwent extensive conformational modification upon removal of the zinc. Rabbit carbonic anhydrases I and II induce pronounced asymmetry in acetazolamide. Titration of the resulting change in ellipticity at 272 nm indicated the affinities of these isoenzymes for the inhibitor to be quite similar, with acetazolamide:protein ratios of 0.53 for carbonic anhydrase I and 0.64 for carbonic anhydrase II, corresponding to one-half total net ellipticity change at this wavelength. In contrast, carbonic anhydrase III does not induce ellipticity in acetazolamide, a fact which is attributed to a substantially lower affinity of the zinc in the enzyme for the sulfonamide group of the inhibitor. In difference spectra of the dimer vs. the monomer forms of carbonic anhydrase III, a broad positive band was observed at 283 nm ($[\theta]_M = 7000$) which has been assigned to the single disulfide bond connecting the two monomer subunits. This change in ellipticity difference as a function of disulfide concentration has been correlated with the rate of reduction of the dimer.

Carbonic anhydrase, a well-characterized enzyme which catalyzes the hydration of CO_2 and the dehydration of HCO_3^-, has been found to occur as two major isoenzymes in the erythrocytes and other tissues of numerous species. These are designated carbonic anhydrase I (the low activity form) and carbonic anhydrase II (the high activity form); they are products of separate genes (Tashian, 1977). We have recently characterized a third major isoenzyme present in rabbit muscle which is sufficiently distinct from these two isoenzymes to be designated as carbonic anhydrase III (Register et al., 1978). It is similar to the mammalian type I and II isoenzymes with respect to certain general properties, i.e., molecular weight, sedimentation coefficient, zinc content, and dependence on zinc for activity (Koester et al., 1977; Register et al., 1978). Amino acid analysis and peptide mapping, however, show differences at the level of primary structure. Also, carbonic anhydrase III possesses several properties that are highly unusual for a mammalian carbonic anhydrase. (1) It has low CO_2 hydratase activity (20% of that of the low activity type I enzyme and 3% that of the high activity type II enzyme), and proportionately even poorer esterase activity. (2) It is comparatively resistant to the highly specific carbonic anhydrase inhibitor acetazolamide, requiring more than 2000 times as much inhibitor for 50% inhibition of CO_2 hydration than the other two isoenzymes. (3) It is immunochemically distinct from carbonic anhydrases I and II. (4) It can exist as a dimer which is the disulfide oxidation product of the monomer and which is maintained by a single disulfide bond linkage.

We have been interested in whether some of these properties of carbonic anhydrase III are related to differences of its conformation in solution as compared with isoenzymes I and II. The present study uses circular dichroism spectrometry of rabbit muscle carbonic anhydrase III in comparison with rabbit erythrocyte carbonic anhydrases I and II to address this question. The effects of pH, relative zinc content, dithiothreitol, and acetazolamide on the spectra of carbonic anhydrase III will be described and their mechanistic and phylogenetic implications discussed.

Materials and Methods

Rabbit blood carbonic anhydrases I and II were prepared according to the method of Walther et al. (1977), rabbit muscle carbonic anhydrase III as previously described (Register et al., 1978). The monomer form (mol wt, 29 000) and the dimer form (mol wt, 58 000) were identified when required by their previously established elution positions on Sephadex G-75 chromatography and by titration with *p*-mercuribenzoate (5 free –SH groups per 29 000 mol wt for the dimer and 6 free –SH groups per 29 000 mol wt for the monomer). Acetazolamide was purchased as Diamox from Lederle Laboratories.

All spectra were taken in 25 mM sodium phosphate buffer of the appropriate pH except for those at pH values of 4.9 and 5.5, where 100 mM sodium succinate was used in order to duplicate experimental conditions previously employed in the removal of zinc (Koester et al., 1977). Dithiothreitol at a concentration of 1 mM was present in all samples except in those of the rabbit muscle carbonic anhydrase III dimer. For the preparation of apoenzyme, the sample was dialyzed for 6 h against 10 mM dipicolinic acid in 100 mM sodium phosphate (pH 6.9) with 1 mM dithiothreitol according to the method of Hunt et al. (1977).

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Table I: Comparison of Maximum and Minimum Molar Ellipticities of the Carbonic Anhydrase Isoenzymes from Various Species

carbonic anhydrase I				carbonic anhydrase II						carbonic anhydrase III	
rabbit		human[a]		rabbit		human[a]		bovine[b]		rabbit	
λ (nm)	$[\theta]_M$ ($\times 10^{-4}$)	λ (nm)	$[\theta]_M$ ($\times 10^{-4}$)	λ (nm)	$[\theta]_M$ ($\times 10^{-4}$)	λ (nm)	$[\theta]_M$ ($\times 10^{-4}$)	λ (nm)	$[\theta]_M$ ($\times 10^{-4}$)	λ (nm)	$[\theta]_M$ ($\times 10^{-4}$)
293	−0.74	295	−1.61	293	−1.40	295	−1.63	296	−1.43	293	−1.05
285	−1.02	285	−2.69	285	−2.62	286	−2.51	284	−1.78	286	−0.24
271	−0.65	275	−3.75	269	−4.17	270	−5.20	276	−1.96	269	−1.36
242	+6.20	246	+8.30[c]	249	+2.59	249	+4.95	247	+5.50	245	+5.85[c]
		232	+15.00							232	+11.30
										222	+48.70
216	−23.30	215	−52.20	214	−81.30	216	−80.60	214	~−80		

[a] Calculated from Beychok et al. (1966) by multiplying their $[\theta]_{MRW}$ values by 260 (the average number of carbonic anhydrase residues). [b] From Coleman (1968). The value at 214 nm was estimated from a graphical presentation. [c] Estimate of the maximum of the shoulder in the area from 240 to 250 nm.

Ultraviolet circular dichroism spectrometry was performed on a Cary Model 60 spectropolarimeter with a Model 6002 circular dichroism attachment, calibrated with *d*-camphor-10-sulfonic acid according to the manufacturer's instructions. Spectra, including base lines, were scanned two to five times at dynode voltages which were always less than 600 V. The same 10-mm path length fused quartz cell was used throughout, with protein concentrations of about 0.5 mg/mL in the 320–230-nm range and 0.1 mg/mL in the 250–200-nm range. The actual protein concentrations were determined from their absorption at 280 nm with $\epsilon_{280}^{0.1\%}$ values of 1.82, 1.74, and 2.32 for carbonic anhydrases I and II (Walther et al., 1977) and carbonic anhydrase III (Register et al., 1978), respectively. Circular dichroism is expressed in units of molar ellipticity, $[\theta]_M$, with molecular weight values of 30 000 for carbonic anhydrases I and II and 29 000 for carbonic anhydrase II employed in the calculations. The units are in degrees per square centimeter per decimole, and the mean residue weight values can be calculated by dividing the $[\theta]_M$ values by 260.

Results

Comparison of Carbonic Anhydrase III with the Erythrocyte Isoenzymes I and II. The circular dichroic spectra of the three major carbonic anhydrase isoenzymes from rabbit are shown in Figure 1, and the major ellipticity bands compared with those found in other species in Table I. In the near ultraviolet, the bands which contribute to the spectra show overall similarity in their λ_{max} values and in their signs, although their intensities vary considerably among both species and isoenzyme types. The negative band at 293 nm varies the least, with much more variation in intensity being found at 285 nm. Both λ_{max} and intensity vary considerably in the band around 270 nm with its associated fine structure. Strong positive dichroism is consistently observed in all isoenzymes and species in the 240–250-nm region. In the rabbit muscle type III and the human type I enzyme, these absorbances are overlapped by the much more intense positive band at 232 nm and are thus visible only as shoulders.

The most distinctive differences among the isoenzymes are found in the far ultraviolet. Carbonic anhydrase III is distinguished by a very intense positive band at 222 nm. The transition which gives rise to this band is not unique to the type III isoenzyme. It is also reflected by a distinct superimposed band at 223 nm in the rabbit type I isoenzyme and by a slight shoulder observed in human carbonic anhydrase I and, to a lesser extent, in human carbonic anhydrase II (Beychok et al., 1966). However, its strong positive intensity appears to be unique for carbonic anhydrase III. In addition, rabbit carbonic anhydrase III shows a less intensive positive

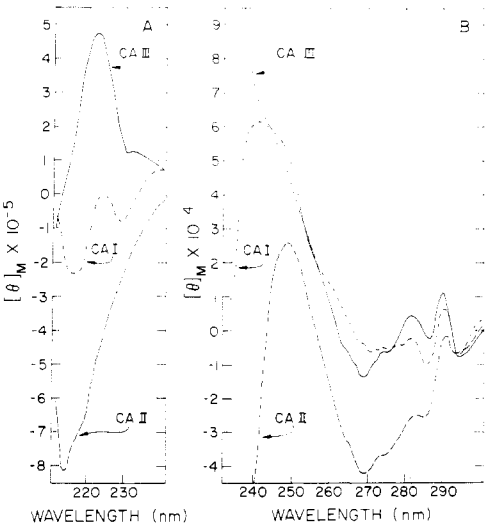

FIGURE 1: Comparison of the circular dichroic spectra of rabbit muscle carbonic anhydrase III with those of rabbit erythrocyte carbonic anhydrases I and II. The conditions are described in the text. Carbonic anhydrase I (CA I, ····); carbonic anhydrase II (CA II, - - -); carbonic anhydrase III (CA III, —). (A) Far-ultraviolet spectra; (B) near-ultraviolet spectra.

ellipticity at 232 nm, which is present in human carbonic anhydrase I but absent in rabbit carbonic anhydrase I. Carbonic anhydrase II isoenzymes in all species examined so far show a pronounced negative band in the area of 215 nm of consistently reproducible intensity, which has been assigned to peptide bond transitions characteristic of the β-pleated sheet conformation (Beychok et al., 1966; Coleman, 1973) dominating the carbonic anhydrase tertiary structure. In carbonic anhydrase I, the contributions from positive bands result in considerable variability in the negative ellipticity in this area, while, in carbonic anhydrase III, the negative contribution is entirely cancelled out.

Effect of Acidic pH on Carbonic Anhydrase III. The results obtained for circular dichroic spectra of carbonic anhydrase III exposed to acidic conditions are shown in Figure 2. At pH 5.5, the spectrum remains unaltered from that obtained at neutral pH for at least 10 h, including the bands at 232 and 245 nm. If these conditions are maintained for several weeks, modification occurs with the band at 232 nm greatly reduced and the other near-ultraviolet ellipticities at wavelengths less than 285 nm somewhat altered. The protein, nonetheless, maintains a considerable amount of structure at this pH, even over extended time periods. Lowering the pH to 4.9 results in a similar transformation which occurs much more rapidly. Note that the band at 232 nm has disappeared entirely after 8 h. Exposure to pH 1.8 for 2 min eliminates all the char-

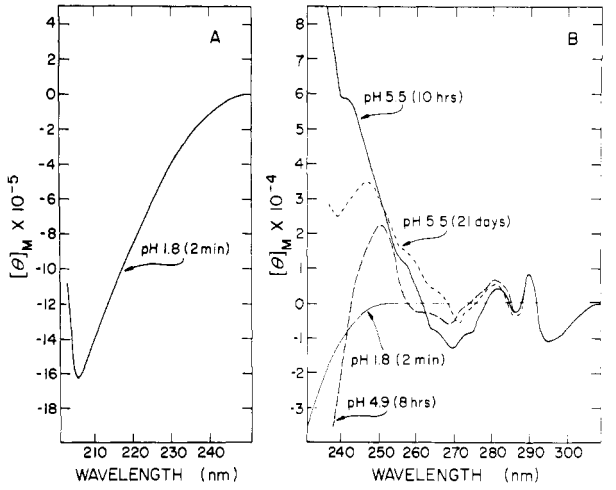

FIGURE 2: Circular dichroic spectra of rabbit muscle carbonic anhydrase III at acidic pH values. The conditions are described in the text. (A) Far-ultraviolet spectra at pH 1.8. (B) Near-ultraviolet spectra. pH 1.8, 2 min (—); pH 4.9, 8 h (- - -); pH 5.5, 21 days (····); and pH 5.5, 10 h (—).

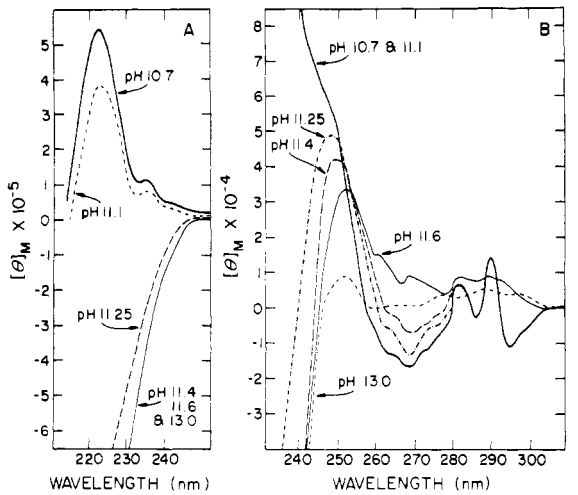

FIGURE 3: Circular dichroic spectra of rabbit muscle carbonic anhydrase III at basic pH values. All spectra shown were taken after at least 15 h of incubation at the indicated values; other conditions are described in the text. (A) Far-ultraviolet spectra. pH 10.7 (—); pH 11.1 (····); pH 11.25 (- - -); and pH 11.4, 11.6, and 13.0 (—). (B) Near-ultraviolet spectra. pH 10.7 and 11.1 (—); pH 11.25 (····); pH 11.4 (- - -); pH 11.6 (—), and pH 13.0 (-•-•).

acteristic ellipticity bands of carbonic anhydrase III and results in the appearance of an intense negative band centered at about 205 nm. Negative ellipticity of this order of magnitude is regarded as representing peptide bond associated transitions characteristic of random coil structure (Adler et al., 1973). Carbonic anhydrases I and II yielded identical spectra at this pH, which is at variance with the results of Beychok et al. (1966) who observed negative bands in the area of 220 nm in human carbonic anhydrases I and II and concluded that acid denaturation induced α-helix formation in these proteins had occurred, but it is in agreement with the results of Wong & Hamlin (1974) on bovine carbonic anhydrase II.

Effect of Basic pH on Carbonic Anhydrase III. As shown in Figure 3, the structure of carbonic anhydrase III is essentially retained up to pH 11.1, although some reduction in the positive bands at 222 and 232 nm occurs. In the near ultraviolet, increasing the pH above this value results in a slight red shift and diminution of intensity in the negative bands around 270 nm. The bands at 293 and 285 nm are maintained unaltered up to pH 11.6, at which point they disappear entirely.

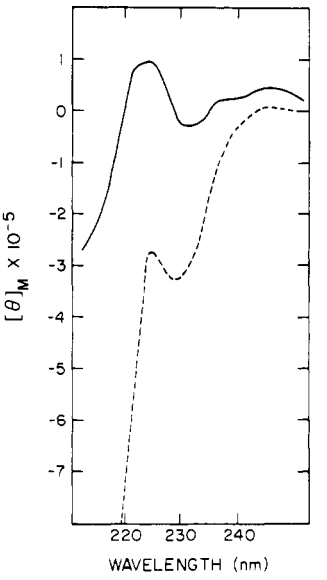

FIGURE 4: Far-ultraviolet circular dichroic spectra of modified rabbit muscle carbonic anhydrase III. Sample lyophilized and dissolved in neutral buffer. The material tends to precipitate and was centrifuged immediately before taking the spectrum and determining the specific activity (—); sample with >90% of zinc removed as described in the text (- - -).

The positive ellipticity remaining at these wavelengths can be attributed almost exclusively to the cystine contribution of the dimer form of carbonic anhydrase III (see below). The band at 245 nm is shown to be diminished and strongly red shifted with increasing pH, and to be present up to pH 13. The red shift (Adler et al., 1973) and the pK_a of 11.4 of this peak suggest that tyrosine is responsible for this ellipticity (Koester, 1979). In contrast, the positive bands at 222 and 232 nm are not red shifted either with increasing pH or with time. A time course experiment (Koester, 1979) shows that they decrease in ellipticity at similar rates, suggesting that the same conformational modification of the protein is responsible for the elimination of both bands. The process is accelerated with increasing pH.

Effect of Zinc Removal or Lyophilization on the Conformation of Carbonic Anhydrase III. Figure 4 shows the far-ultraviolet circular dichroic spectrum of a sample of carbonic anhydrase III in which the zinc was removed at neutral pH. Large amounts of protein were precipitated during this process; the spectrum is of the material that remained in solution after centrifugation. The band at 232 nm is eliminated and the one at 222 nm is greatly reduced. The near-ultraviolet spectrum (not shown) is similar to the acid denaturation spectra presented above, with reduction of the bands at 245 and in the area of 270 nm and the retention of those at 285 and 293 nm. The addition of up to 0.1 M $ZnSO_4$ to either holoenzyme or apoenzyme has no effect on their respective ellipticities.

The far-ultraviolet spectrum of a sample of carbonic anhydrase III which had been lyophilized and stored over long periods of time at −10 °C is also shown in Figure 4. Enzyme kept in this manner and remaining in solution when dissolved in neutral buffer retains full activity as a CO_2 hydratase. It is distinguished in conformation from enzyme not so treated by the marked diminution of its positive ellipticity at 222 and 232 nm, even though the near-ultraviolet bands (including the positive one at 245 nm) remain intact.

Effect of Acetazolamide on the Spectra of Carbonic Anhydrases I, II, and III from Rabbit. The effect of a 1.5 M excess of acetazolamide on the three isoenzymes is shown in

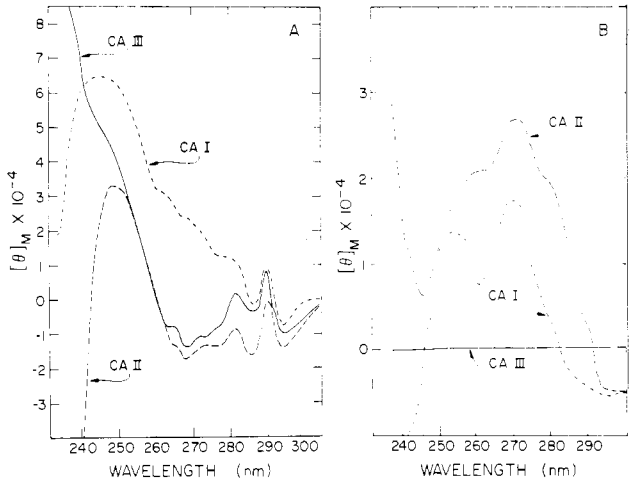

FIGURE 5: Effect of acetazolamide on the circular dichroic spectra of rabbit erythrocyte carbonic anhydrases I and II and rabbit muscle carbonic anhydrase III. Carbonic anhydrase I (CA I, ····); carbonic anhydrase II (CA II, - - -); and carbonic anhydrase III (CA III, —). The pH was 7.0 and acetazolamide was present in a 1.5 M excess. (Note: Acetazolamide itself does not display any ultraviolet circular dichroism.) (A) Spectra of the enzyme:acetazolamide complexes. (B) Difference spectra derived from the subtraction of the native protein spectra from those shown in part A.

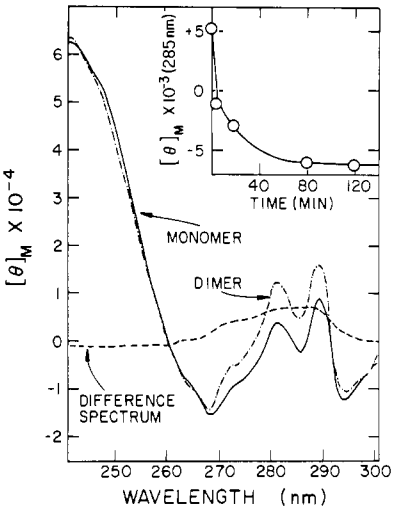

FIGURE 6: Near-ultraviolet circular dichroic spectra of monomer and dimer forms of rabbit muscle carbonic anhydrase III. The spectra were taken at pH 7.0 with 1 mM dithiothreitol present for the monomer and no dithiothreitol for the dimer. Monomer (—); dimer (····); and difference spectrum (- - -). The inset figure shows the time course of the dimer–monomer conversion followed at 285 nm. A scan was performed on the dimer species to establish a base-line value, and an equivalent amount of protein was injected into buffer containing 15 mM dithiothreitol followed by scans at the indicated time points.

Figure 5A and the resulting difference spectra in Figure 5B. The latter are concluded to originate primarily from the asymmetry which is induced in acetazolamide by carbonic anhydrases I and II rather than vice versa because (a) the maximum of the difference spectra is close to 267 nm, which is the absorption maximum of acetazolamide at pH 7 (Lindskog, 1968; Koester & Noltmann, unpublished results), and (b) the fine structure features observed in the 270-nm region of the native proteins are also observed in their complexes with acetazolamide. However, contributions from Tyr-194 or Trp-209 cannot be unequivocally ruled out. Both residues can contribute ellipticity in this region (Adler et al., 1973), and both are invariant constituents of the hydrophobic side of the active site cleft of carbonic anhydrases I and II (Nostrand et al., 1975). In both isoenzymes Trp-209 interacts closely with the aromatic portion of acetazolamide (Kannan et al., 1976).

Titration of carbonic anhydrases I and II with increasing amounts of acetazolamide yielded hyperbolic saturation curves from which values for the acetazolamide:protein ratio at which half of the total ellipticity change has occurred, of 0.53 for carbonic anhydrase I and 0.64 for carbonic anhydrase II, were calculated (Koester, 1978). These are both very close to the ratio of 0.56 at which half-maximal inhibition of both the rabbit erythrocyte isoenzymes has been observed (Walther et al., 1977). The difference spectra indicate that the I and II isoenzymes have different acetazolamide environments, but the titration data show that their affinities for the inhibitor are the same. This is in marked contrast to the results obtained for carbonic anhydrase III, which induces no asymmetry whatsoever in acetazolamide. It is not possible to raise the inhibitor levels sufficiently to see an effect, because its absorbance in this region results in unacceptably high background noise at higher concentrations.

Circular Dichroism of Monomer and Dimer Forms of Carbonic Anhydrase III. We have previously shown that rabbit muscle carbonic anhydrase III, in the absence of reducing agents, can form a dimer with a single intermolecular disulfide bond between the two monomers (Register et al., 1978). The circular dichroic spectra of the two species are shown in Figure 6. The difference spectrum is characterized by a broad positive band centered at about 284 nm with $[\theta]_M = 7000 \pm 200°$ cm^{-2} dmol^{-1}. This spectrum is consistently reproducible whether the monomer was initially in that configuration or produced by adding dithiothreitol to the dimer species. The half-time of the latter process is about 5 min, a value which is in a range previously observed for proteins with accessible disulfide bonds (Iyer & Klee, 1973). It has previously been pointed out that the equilibrium favors the monomer, and that the conversion of monomer to dimer is slow (25% conversion in 2 weeks) under physiologically relevant conditions (Register et al., 1978). Bewley (1977a) has shown that neither oxidized nor reduced dithiothreitol by itself has circular dichroism in the ultraviolet. He has also argued convincingly (1977b) that, even if the protein dithiothreitol intermediate were optically active, the protein if quantitatively reduced should be free from optical activity of mixed disulfide, and that therefore the rate of ellipticity change, although it may not reflect the exact kinetics of reduction, should be in the right range.

The spectrum of carbonic anhydrase III at pH 13 (Figure 3) shows a positive band centered in the area of 282 nm which is similar in sign and wavelength albeit somewhat less in intensity than the difference spectrum band. It has previously been found (Koester et al., 1978) that isoelectric focusing of carbonic anhydrase III in a basic pH environment leads to accelerated generation of the dimer species. Also, pH values above 8.5 have been shown to strongly promote the autooxidation of dithiothreitol (Misra, 1974), thus rendering it a less effective reducing agent and making dimer formation more likely. Therefore, the presence of this ellipticity suggests that the disulfide bond can be maintained intact even when most of the secondary and tertiary structures of the protein have been lost. It also confirms our earlier observation that basic pH promotes dimer formation (Koester et al., 1978).

Discussion

Analysis of the Circular Dichroic Spectrum of Carbonic Anhydrase III. The contributions of tryptophan (Strickland et al., 1969) and tyrosine (Horwitz et al., 1969) to the near-ultraviolet circular dichroism spectra of proteins have

been described. They have been shown to account for the ellipticities observed in this region for carbonic anhydrases I and II (Beychok et al., 1966; Coleman, 1973). A similar explanation is applicable to carbonic anhydrase III as well except that in the range from 270 to 279 nm we are inclined to attribute them more to tryptophan effects than to effects originating from tyrosine since we have observed comparatively little red shifting. The more pronounced red shift at the positive 245-nm band, on the other hand, indicates that there the tyrosine contributions are more important. The positive bands at 222 and 232 nm are not red shifted at all and can therefore be most plausibly attributed to tryptophan. Their intense positive circular dichroism is notable in that only small effects of that sign have been observed for some low activity carbonic anhydrases (type I) and, as a rule, are quite rare for proteins of any kind.

Disulfide contributions to circular dichroic spectra have been reviewed by Adler et al. (1973) and by Beychok (1965). Their near-ultraviolet bands are broad and weak compared with those by aromatic residues. Moreover, as pointed out by Woody (1973), the sign and intensity of the disulfide ellipticity (but not its λ_{max}) can be considerably altered if a strong coupling center is close to the disulfide moiety. In the absence of information about such centers, it is not possible to provide a definitive interpretation of the cystine contributions to the spectrum of carbonic anhydrase III.

In terms of its overall features carbonic anhydrase III is qualitatively similar to its isoenzymes. Features other than differences in its gross tertiary structure determining its circular dichroism (which appears to be highly conserved) must therefore account for its unusual kinetic behavior. It is significant that low activity carbonic anhydrase I enzymes have substantially greater positive ellipticity in the far ultraviolet than the high activity carbonic anhydrases II. Carbonic anhydrase III has an even greater positive contribution in this region, thus raising the possibility that a tendency toward such contributions can be positively correlated with lowered activity in carbonic anhydrases. In that respect, therefore, carbonic anhydrase III has more in common with the low activity carbonic anhydrase I. Interestingly, carbonic anhydrase III has more resemblance to carbonic anhydrase II with respect to its primary structure (Register et al., 1978). The near-ultraviolet spectra of all carbonic anhydrases indicate that their secondary and tertiary structures have been highly conserved throughout evolution. In fact, data for the shark enzyme have been interpreted to indicate that this conservation has held for at least 400 million years (Maynard & Coleman, 1971).

Stability of Carbonic Anhydrase III. Carbonic anhydrase III retains the essential features of its circular dichroic spectrum in the pH range 5–11, with some alterations at the extremes of this range observed if the sample is stored under those conditions for extended periods of time. In this respect carbonic anhydrase III is again more similar to carbonic anhydrase I than to carbonic anhydrase II, having in common with it a greater stability in base compared with that observed for the type II enzyme (Beychok et al., 1966; Riddiford et al., 1965).

A unique feature of carbonic anhydrase III, however, is the extent to which zinc contributes to the stability of the tertiary structure of the enzyme. In early studies of the conformational changes in carbonic anhydrase by optical rotatory dispersion (Coleman, 1965; Lindskog & Malmstrom, 1962), it was concluded that acidic conditions were necessary for the removal of zinc by 1,10-phenanthroline. When changes in the optical rotatory dispersion spectra were not observed upon removal of the zinc, it was concluded that the apoenzyme and the holoenzyme had the same tertiary structure. Subsequently, circular dichroism was employed in studies of the relative susceptibilities of bovine holo- and apocarbonic anhydrase II to guanidine hydrochloride denaturation (Wong & Hamlin, 1975; Yazgan & Henkens, 1972). The apoenzyme was again shown to be similar in conformation to the holoenzyme, although somewhat more susceptible to denaturation. It was also found that zinc was not required for renaturation, although it accelerated its rate and influenced the pathway of the process. These data concerning the conformation of carbonic anhydrase in solution agree with the crystal structure results showing that the apoenzyme is isomorphous with the holoenzyme (Kannan et al., 1971). Hunt et al. (1977) have recently described a method for the removal of zinc from carbonic anhydrase at neutral pH, which has enabled us to test the relative effects of acidity and zinc removal on the structure of carbonic anhydrase III. Our results demonstrate clearly that incubation at pH 5.5 does not disrupt the structure of carbonic anhydrase III if zinc is present (except over greatly extended time periods), but that the removal of zinc at neutral pH produces extensive modification. Preliminary results on the sequence of bovine muscle carbonic anhydrase II (Tashian et al., 1978) show that the histidine residues which are zinc ligands in carbonic anhydrases I and II (94, 96, and 119) are conserved in carbonic anhydrase III; therefore, other residues in the neighborhood of the zinc must be responsible for its unusual role in isoenzyme III.

Effect of Acetazolamide on the Carbonic Anhydrase Isoenzymes. Initial observations of the effects of acetazolamide on carbonic anhydrases I and II with optical rotatory dispersion yielded no significant differences between the enzymes themselves and the enzyme–inhibitor complexes (Coleman, 1965; Armstrong et al., 1966). The more sensitive circular dichroism technique was used subsequently to observe changes induced by these isoenzymes in azo-dye derivatives of sulfonamides at both visible and ultraviolet wavelengths (Coleman, 1968). Circular dichroism has not been previously employed, however, to specifically calculate differences in sulfonamide affinity between carbonic anhydrase isoenzymes. In applying this technique we found the affinities of carbonic anhydrases I and II for acetazolamide to be the same which is somewhat at variance with kinetic inhibition data reported for other species. Thus, McIntosh (1969) found that the rat type I enzyme is more strongly inhibited by acetazolamide; conversely, Verpoorte et al. (1967) had reported that the human type II enzyme was more powerfully inhibited by a factor of ten. Kannan et al. (1976) have attributed the latter difference to several hydrophobic interactions, especially that of residue 121 (valine in type II and alanine in type I enzyme species) with the sulfur of the acetazolamide ring. However, this residue is alanine only in human carbonic anhydrase I of all the type I and II carbonic anhydrases so far sequenced (Tashian, 1977). Therefore, the substitution cannot be regarded as typifying a general type I vs. type II difference with respect to acetazolamide affinity.

In order to account, then, for the very striking difference between carbonic anhydrase III and the other two isoenzymes with regard to acetazolamide binding, one might invoke (a) differences in the hydrophobic interactions in the active site with the aromatic ring of the inhibitor, or (b) differences in the coordination of the sulfonamide group itself to the zinc. Residues 91, 121, and 131 are regarded (Kannan et al., 1976) as the most critical with respect to the first hypothesis. Residue 91 is quite variable (but always hydrophobic), residue 121 is

valine (except in human carbonic anhydrase I), and residue 131 is leucine in type I enzymes and phenylalanine in type II enzymes. The partial sequence of bovine carbonic anhydrase III (Tashian et al., 1978; R. E. Tashian, personal communication) indicates that hydrophobicity is conserved for two of these three residues, which for this species are arginine at position 91, valine at 121, and leucine at 131. Furthermore, the apoenzyme forms of carbonic anhydrases I and II, with the hydrophobic groups assumed to be still available for interaction with the inhibitor, have a binding constant of $\sim 10^{-4}$ M (Coleman, 1967; King & Burgen, 1976) which is of the same order of magnitude as that which we have observed for carbonic anhydrase III (Koester et al., 1977).

As noted above, changes in the hydrophobic residues cause variable (if any) differences in the affinity of acetazolamide for enzymes I as compared with II, which are no larger than one order of magnitude. In contrast, metal ion substitutions can produce drastic changes in the acetazolamide affinity for these enzymes (K_d ranging from 9×10^{-4} to 7×10^{-8} M) caused almost entirely by changes in the off-rate (Harrington & Wilkins, 1977). Also, Taylor et al. (1970) have concluded from enthalpy calculations that the sulfonamide interaction is primarily stabilized by a dominant metal–ligand bond and only secondarily by hydrophobic interactions. Their kinetic results support a mechanism of sulfonamide inhibitor binding to carbonic anhydrase consisting of two steps, the first being an interaction of the inhibitor with the hydrophobic residues at the active site, and the second the formation of a tight zinc–sulfonamide group linkage with a low off-rate.

The presence of arginine at a critical hydrophobic site might inhibit the first step. The second step could conceivably be inhibited as well since our results show significant differences between carbonic anhydrase III and the other two isoenzymes with respect to zinc-protein interaction. Further experimentation with carbonic anhydrase III and other acetazolamide-resistant carbonic anhydrase species will be necessary to ascertain the relative contributions of each to the events in the mechanistic sequence.

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