A comparative CD study of carbonic anhydrase isoenzymes with different number of tryptophans: Impact on calculation of secondary structure content

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(RECEIVED June 7, 1996; ACCEPTED September 25, 1996)

Abstract

The CD spectra of human carbonic anhydrase I and II and bovine carbonic anhydrase III were recorded and analyzed. The 3D structures of these isoenzymes are known, showing very similar secondary structure and polypeptide-chain fold. The tryptophan content, however, differs between the isoenzymes, i.e., isoenzymes I, II, and III possess 6, 7, and 8 tryptophans, respectively. All of the tryptophans except the additional tryptophans in isoenzymes II and III, i.e., W245 and W47, are conserved. Despite the fact that X-ray structure determinations showed that the isoenzymes had highly similar secondary structure, the contents of α -helix and β -sheet structure differed considerably when using different CD algorithms for estimation of the fractions of various secondary structural elements. This shows that aromatic amino acids also interfere in the wavelength region (far-UV) used to calculate the amount of secondary structure. Such interference is especially problematic when analyzing proteins like carbonic anhydrase, which consist mainly of β -structure that gives rise to weak ellipticity bands, compared to the bands arising from α -helical structure.

Keywords: aromatic amino acids; bovine carbonic anhydrase III; β -structure; human carbonic anhydrase I and II

Proteins exhibit characteristic CD spectra in the far-UV region and the appearance of these spectra are related to the presence of secondary structure (Greenfield & Fasman, 1969). Accordingly, CD is frequently used to estimate the fractions of various types of secondary structure in proteins (Saxena & Wetlaufer, 1971; Chen et al., 1972; Provencher & Glöckner, 1981; Compton & Johnson, 1986; Manavalan & Johnson, 1987; Perczel et al., 1991). Knowledge of the secondary structure is of great importance in the understanding of the function, stability, and folding of a protein, and CD estimations of the amount of α -helices have been particularly accurate for many proteins. On the other hand, it has been more difficult to predict the contents of β -structure (Sreerama & Woody, 1994). One obvious reason for this is that the α -helix spectrum dominates the protein far-UV CD spectrum (Johnson, 1990; Sreerama & Woody, 1993), and the CD signal arising from β -structure is of lower intensity. This means that calculations of the amount of this type of secondary structure are more sensitive to perturbations from other contributing structural elements. Based on theoretical calculations and experimental evidence from peptide models, it has been suggested that aromatic amino acids make contributions to the far-UV CD spectrum (Strickland, 1974; Man-

ning & Woody, 1989; Manning et al., 1992). Because this wavelength region is used for estimation of the amount of secondary structure and the models used do not consider aromatic contributions explicitly, aromatic amino acids could seriously interfere with such calculations.

In a recent study, we used site-directed mutagenesis to replace one Trp at a time in human carbonic anhydrase II (HCA II) in order to assign the contribution of each of the Trp residues to the CD spectrum (Freskgård et al., 1994). We found that the individual Trp residues contributed to the CD spectrum not only in the near-UV wavelength region, but also in the far-UV region. Because the latter contribution was substantial in the far-UV region, Trp residues may interfere significantly with predictions of the amount of various types of secondary structure. Moreover, proteins that, like carbonic anhydrase, contain several Trp residues and a dominating β -structure that gives rise to low-intensity CD bands, might be especially sensitive to such interference.

To further characterize the impact of Trp residues on the CD spectrum and on calculations of the amounts of secondary structure, we investigated the CD spectra obtained from three different isoenzymes (I–III) of carbonic anhydrase, i.e., HCAI and II, and bovine CAIII (BCAIII), for which the 3D crystal structures are known. The X-ray structure determinations indicate that these isoenzymes are essentially identical with respect to tertiary and secondary structures (Kannan et al., 1975; Eriksson et al., 1988; Håkansson et al., 1992; Eriksson & Liljas, 1993), but differ in

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regard to Trp content. Specifically, isoenzymes I, II and III contain six, seven, and eight Trp residues, respectively; the six Trp residues in HCAI are also present in the other two isoenzymes studied, and the seventh Trp in HCAII is conserved in BCAIII (Andersson et al., 1972; Henderson et al., 1976; Hewett-Emmet & Tashian, 1991). Considering the fact that these isoenzymes have almost identical secondary structure, comparisons of their CD spectra should shed some light on the significance of interference by specific Trp residues in the spectrally based prediction of secondary structure content.

Results and discussion

Structural description

Crystal structure determinations of the three carbonic anhydrase isoenzymes HCAI, HCAII, and BCAIII have revealed very similar secondary structure and polypeptide-chain fold (Fig. 1) (Kannan et al., 1975; Eriksson et al., 1988; Håkansson et al., 1992; Eriksson & Liljas, 1993).

Mutagenesis of Trp residues in HCAII led to significant changes in the CD spectrum both in the near- and the far-UV wavelength region (Freskgård et al., 1994). This indicates that differences in Trp content in the isoenzymes can have a marked impact on the CD spectra of the carbonic anhydrases. HCAI contains six Trp residues, all of which are found, in the same sequence positions, in HCAII and BCAIII. HCAII has seven Trp residues; the extra Trp, i.e., W245, is also present in BCAIII, but is replaced by Asn in HCAI. In BCAIII, an additional Trp (W47) is found, leading to a total of eight Trp residues in this isoenzyme. In this position, Ile and Leu are present in HCAI and II, respectively (Andersson et al., 1972; Henderson et al., 1976; Hewett-Emmet & Tashian, 1991).

CD spectra of HCAI and HCAII

The CD-spectra of the three isoenzymes are shown in Figure 2A and B. CD-spectra of HCAI and II have been published previously (Beychok et al., 1966; Carlsson et al., 1975; Freskgård et al., 1994), and the CD spectrum of HCAI is in this work extended to 180 nm in the far-UV region. The spectra in Figure 2 agree very well with those shown in the cited studies. According to Beychok et al. (1966), description of the CD spectra of HCAI and II in the near-UV wavelength region requires at least two major CD bands and at least two more small bands to account for the fine structure above 280 nm. However, in the CD spectrum of HCAII, there appears to be an additional band at 278 nm (Freskgård et al., 1994) and the 275-nm band in HCAI (Beychok et al., 1966) was resolved into two bands with negative ellipticities, 272 and 278 nm (Table 1). From 270 to 296 nm, six bands with the same wavelength extrema, or with extrema differing by just 1 or 2 nm, can be seen in the spectra of both isoenzymes, although the ellipticities of the bands differ (Table 1). Thus, the near-UV CD spectrum of HCAI, like that of HCAII, is unusually complex, probably because the L_a band can couple strongly with the B_b bands of nearby Trp residues, and with strong far-UV bands of Tyr and Phe. An additional source of complexity in the near-UV spectrum is the heterogeneity of the environment, which affects the L_a band positions especially. In the far-UV region (180–220 nm), three main bands can be distinguished for both HCAI and HCAII (Fig. 2B; Table 1), although the magnitudes of the bands differ. A positive band at ~ 200 nm and two negative bands at ~ 180 nm and 205–220 nm, respectively, are typical for β -sheet structure (Manning & Woody, 1987), and we found that HCAI exhibited these spectral features. This means that the CD spectra of HCAI imply qualitative agreement with the crystal structures of the enzymes in which β -structure is predominant.

Fig. 1. Alignments of the polypeptide backbones of HCAI (red), HCAII (blue), and BCAIII (green). When HCAI and HCAII, HCAI and BCAIII, HCAII and BCAIII are superimposed, the following RMS deviations for all the backbone atoms in aligned positions are obtained: 1.0 Å, 0.91 Å, and 0.87 Å, respectively.

Fig. 2. A: Near-UV CD spectra of HCAI (-----), HCAII (—), and BCAIII (— · —). B: Far-UV CD spectra of HCAI (-----), HCAII (—), and BCAIII (— · —).

CD spectra of BCAIII

The far-UV CD spectrum (180–220 nm) of BCAIII is also characteristic of a β -sheet protein and is qualitatively similar to the corresponding spectral region of HCAI and II, although the intensities of the bands differ (Fig. 2B; Table 1). All bands that are detected in the wavelength region 260–320 nm of the near-UV CD spectra of HCAI and II can also be seen in the spectrum of BCAIII (Fig. 2A). However, in the latter, the magnitudes of the spectral minima are significantly smaller, and the maxima at 283 and 291 nm even have positive ellipticities (Table 1).

CD spectrum of individual W47

It has been claimed that Trp residues are the major determinant of the near-UV CD spectrum (Strickland, 1974) and our recent study of Trp mutants of HCAII supports this suggestion, because the Trp residues were shown to be responsible for the main part of the CD spectrum in this spectral region (Freskgård et al., 1994). Thus, the

extra Trp residue, i.e., W47, most likely is mainly responsible for the aberrant appearance of this part of the spectrum. A tentative individual spectrum of W47 is obtained (Fig. 3) by calculating the difference CD spectrum between the spectra of BCAIII and HCAII; this occurs because HCAII contains all Trp residues that are present in BCAIII, except W47. The contributions of W47 in the far-UV region have not been determined because the different contents of the other aromatic amino acids of the isoenzymes are considered to interfere with such a calculation (Woody, 1978; Vuilleumier et al., 1993).

Orientations and surroundings of the Trp residues

The spectra of the three isoenzymes are similar in shape, indicating that the six Trp residues in common are sensing a similar environment that is further indicated by comparisons of the localizations of the Trp residues in the 3D structure of carbonic anhydrase. All indol rings of the Trp residues that the three isoenzymes have in common are oriented very similarly relative the polypeptide

Table 1. Wavelength extrema and intensities of the CD spectra from HCAI, HCAII, and BCAIII^a

HCAI		HCAII		BCAIII	
λ (nm)	$[\theta]^a$	λ (nm)	$[\theta]^a$	λ (nm)	$[\theta]^a$
183	-12,000	181	-8,900	181	-12,700
187	-12,200			189	-9,350
202	1,160	201	600	202	22,10
211	-2,130	211	-3,660	209	-1,070
237	468	246	186	225	1,540
272	-133	270	-236	269	-55
278	-143	278	-195	278	-6
283	-105	284	-127	283	38
286	-129	287	-147	287	-15
292	-8	291	-44	291	51
295	-58	296	-95	295	-48

^aMean residue molar ellipticity.

Fig. 3. Contribution of Trp 47 to the near-UV CD spectrum of BCAIII. The spectrum was obtained as a difference spectrum: BCAIII – HCAII.

backbone (Fig. 4). The torsion angles ($C_\alpha C_\beta - C\gamma C_{\delta 1}$) are within $\pm 4^\circ$ in each position, except for W209 in BCAIII, which deviates -5.3° from the average torsion angle. Obviously, the isoenzymes exhibit only rather small structural differences, and they should therefore be suitable for use in theoretical CD calculations.

From inspection of the neighbors, it is also evident that the residues surrounding the Trp residues in the isoenzymes are more highly conserved than in the rest of the molecule. In the whole molecule, the sequence identities between the investigated isoenzymes amount to 43%, whereas residues within 5.5 Å from the six invariant Trp residues are identical to 61%. The distances and angles to the neighboring groups are also in reasonably good agreement, indicating that the Trp residues in the various isoenzymes are located in similar environments.

Secondary structure estimations

Judging from the X-ray structures of HCAI and II and BCAIII, the parts that constitute the secondary structure in the isoenzymes are very similar. According to the refined structures reported for HCAII and BCAIII, the main-chain atoms of the 95 residues that make up the predominant β -sheet were defined to be equivalent and the calculated RMS fit is 0.39 Å (Eriksson & Liljas, 1993). The carbonic anhydrase molecule is divided into two halves by the twisted 10-stranded β -structure. Part of this β -sheet consists of two pairs of parallel chain segments, whereas the rest of the structure is antiparallel. In addition, there are five β -strands as well as some helical structure. All three isoenzymes have these structural features in common.

The contents of the various types of secondary structure of the three isoenzymes are summarized in Table 2. As can be seen, the amount of each secondary structural element, calculated from the X-ray structure, is almost identical in each isoenzyme.

According to the crystal structures, the contents of helical structure for the three isoenzymes range from 15.9 to 18.9%; the corresponding values calculated from the CD spectra by use of two different methods are 3–12% (Manavalan & Johnson, 1987) and 0–8% (Provencher & Glöckner, 1981). According to the X-ray determination, the amount of β -structure in the isoenzymes is between 28.7 and 29.8%, and, again using the two methods just mentioned, calculations based on the CD spectra, result in β -structure contents of 31–44% (Manavalan & Johnson, 1987) and 48–53% (Provencher & Glöckner, 1981). Obviously, the results obtained with X-ray crystallography and CD are not in good agreement, although the correlation is somewhat better when considering the method of Manavalan and Johnson (1987). The poor correlation is not limited to the determined absolute values of the contents of secondary structure, but also applies when comparing the isoenzymes themselves, despite their very similar polypeptide chain

Fig. 4. Alignment of the Trp residues present in HCAII (blue) and BCAIII (green). RMS deviation in aligned positions is 3.4 Å. Numbering indicates the position of each Trp residue in the amino acid sequence.

Table 2. Secondary structure contents in various carbonic anhydrase isoenzymes determined by X-ray and CD

	Helical (%)			β -Sheet (%)		
	X-ray ^a	CD ^b	CD ^c	X-ray ^a	CD ^b	CD ^c
HCAI	18.9	8	0	29.8	31	48
HCAII	16.3	12	8	28.7	38	49
BCAIII	15.9	3	3	29.1	44	53

^aCalculated according to Kabsch and Sander (1983).^bDetermined according to Manavalan and Johnson (1987) in the wavelength region 178–260 nm.^cDetermined according to Provencher and Glöckner (1981) in the wavelength region 190–240 nm.

fold. These deviations must primarily be due to variation in the content of the aromatic amino acid residues that interfere with the far-UV CD bands used for the calculation of secondary structure content.

Besides the spectral effects due to the mentioned different Trp contents in the isoenzymes, variation in amounts of other aromatic residues can also contribute to differences, especially in the far-UV CD spectral region, and thereby influence estimates of secondary structure content. Possibly of particular importance in this context are the differences in the two aromatic clusters present in the protein structure, one small in the N-terminal region and one larger cluster in the central part of the molecule. All of the Trp residues included in the aromatic clusters (nos. 5, 16, and 97) and all of the amino acids in the smaller N-terminal cluster are conserved in the three isoenzymes examined. In HCAI and II, the two larger aromatic clusters are also completely conserved, including seven invariant Phe residues (nos. 66, 70, 93, 95, 176, 179, and 226), besides W97. Four of these seven Phe residues are also found in the larger aromatic cluster in BCAIII, whereas three have been replaced by nonaromatic apolar residues (C66, L95, and L226) (Eriksson & Liljas, 1993). This structural difference should not affect the near-UV CD region significantly, because the intensity of the contribution expected for a Phe compared to that of a Trp is negligible here (Strickland, 1974), although indirect effects by alteration of the environment of the Trp residues cannot be excluded. Replacements of Phe residues in barnase also showed that the Phe residues contributed only modestly or not at all in near-UV CD, but affected the spectrum more predominantly in the far-UV region (Vuilleumier et al., 1993). Thus, for BCAIII, these differences in Phe content might, in addition to the extra Trp, interfere with the peptide CD bands and thereby further obscure the calculations of secondary structure content. Even though there are five Cys residues in BCAIII (Engberg et al., 1985), no disulfide bridges are found in the enzyme, hence no interference from the Cys residues is to be expected.

Concluding remarks

This study emphasizes the impact of aromatic amino acid residues when determining secondary structure content from CD spectra. Thus, the results regarding the fractions of α -helix and β -structure vary considerably when different methods for deconvoluting the CD spectra are used for three carbonic anhydrase isoenzymes with very similar polypeptide folds and content of secondary structure, but different aromatic amino acid composition.

This finding is important, for example when interpreting data obtained in protein folding studies in which folding intermediates are monitored kinetically or at equilibrium by CD measurements, because an alteration of the CD spectrum in the far-UV region can be at least partly due to changes in the local environment around aromatic amino acid residues, i.e., not only to changes in secondary structure.

The three isoenzymes with very similar secondary and tertiary structure, a large fraction of aromatic amino acid residues and low-intensity far-UV CD spectra should be most suitable for theoretical calculations of aromatic contributions to this important region of the CD spectrum.

Also, the possibility to determine individual spectra of different Trp residues is a technique that should be further exploited. For instance, individual Trp CD spectra might give information about local conformational changes during folding. At present, only global conformational changes are mostly monitored by CD. Because the CD spectrum of W47 of BCAIII is the only one that has positive bands in the near-UV region, these peaks might be used to observe when the substructure surrounding this Trp is forming native-like tertiary interactions during the folding process.

Materials and methods

Protein purification

Bovine muscle carbonic anhydrase III was purified from lean skeletal muscle from cattle according to the procedure reported by Borén et al. (1991). The protein was stored cold (4 °C) under nitrogen in a Tris H₂SO₄ buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM DTT. HCAII, pseudowild-type with the substitution C206S, was expressed in *Escherichia coli* BL21(DE3) (Mårtensson et al., 1995) and purified by affinity chromatography (Khalifah et al., 1977). HCAI was prepared as described by Henderson and Henriksson (1973). The purity of the prepared isoenzymes was analyzed by SDS-PAGE.

CD measurements

CD spectra were recorded on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France) calibrated with 10-camphorsulfonic acid (Hennessey & Johnson, 1982). Each spectrum is the average of three scans using a band width of 0.5 nm and an integration time of 2 s. Before summation, the separate scans were compared to detect possible alteration of the sample during the measurements. The spectra were corrected by using a reference solution without protein and then a smoothing algorithm was used with the minimum filter in the CD6 software. The digital low-pass filter with three high-end cut-off frequencies performs the three filtering levels available. The smoothing algorithm reduces all intensity differences between adjacent data points, making the background noise level decrease to a nearly flat level while only slightly reducing the heights of major features. The ellipticity, $[\theta]$, was calculated from

$$[\theta] = [\theta]_{obs}(mrw)/10cl$$

where $[\theta]$ is the mean residue molar ellipticity in deg·cm²·dmol⁻¹, $[\theta]_{obs}$ is the ellipticity in degrees, mrw is the mean residue molecular weight, c is the protein concentration in g/mL, and l is the path length of the cell in cm. All spectra were recorded at 23 °C and

with constant N₂ flushing. The protein solution was filtered (Millipore; pore size, 0.8 µm) and diluted with 10 mM sodium phosphate buffer, pH 7.5, to a concentration of 0.5 mg/mL (17 µM) determined with a Hitachi U2000 spectrophotometer.

Structure analysis

The carbonic anhydrase tryptophans and their surroundings were examined by using the molecular modeling program HyperChem. The coordinates for the atoms of the crystallographic structures of BCaIII and HCAII were kindly provided by Elisabeth Eriksson, Uppsala University, and Kjell Håkansson, Lund University, respectively. The HCAI coordinates were obtained from Brookhaven Protein Data Bank (2cab.pdb).

Acknowledgments

We thank Dr. A. Elisabeth Eriksson, Uppsala University, for providing us with the coordinates of BCaIII as well as calculations of the contents of secondary structure from the X-ray structure. We are also indebted to Göran Aronsson, Umeå University for preparing Figures 1 and 4. This work was supported by grants from the Swedish National Board for Industrial and Technical Development, the Swedish Natural Science Research Council and "Sven and Lily Lawskis fond" (P.-O. F.).

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