Assignment of the Contribution of the Tryptophan Residues to the Circular Dichroism Spectrum of Human Carbonic Anhydrase II†

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ABSTRACT: The circular dichroism (CD) spectrum of human carbonic anhydase II (HCAII) has been investigated using various mutants of the enzyme in which tryptophans have been replaced by site-directed mutagenesis. HCAII contains seven tryptophans which are believed to significantly contribute to the CD spectrum in both the near- and far-UV regions. By substituting the tryptophans one at a time, the spectral effects of the individual tryptophans were studied. The near-UV spectrum of HCAII is very complex, with multiple Cotton effects. This complexity has been attributed to aromatic amino acids, especially tryptophans, located in asymmetric aromatic clusters in the molecule. CD spectra of the individual tryptophans were calculated as difference spectra between the CD spectrum of HCAII and those of the tryptophan mutants. These spectra showed that the tryptophans contributed to the CD spectrum in almost the entire wavelength region investigated (180−310 nm). Summation of the individual tryptophan CD spectra in the near-UV region yielded a spectrum that was qualitatively very similar to that of HCAII, showing that the tryptophans are the major determinant for this part of the CD spectrum. Since tryptophans were also demonstrated to contribute significantly in the far-UV region, tryptophans can interfere considerably with the assignment of changes in CD bands to changes in secondary structure content during folding reactions. Moreover, because of this substantial interference, predictions of the amount of various types of secondary structure from CD data from the far-UV region are made more difficult. These findings are probably of general importance for proteins that, like HCAII, contain several tryptophans. However, considering more specific applications, some of the tryptophans gave rise to unique CD bands that can be utilized to probe the folding process specifically in both kinetic and equilibrium experiments.

Circular dichroism (CD)[1] spectroscopy is often used empirically to monitor conformational changes that occur, for example, during protein folding, and to estimate amounts of the various types of secondary structure. However, the CD spectra of β-proteins having similar secondary structures and little or no α-helix vary widely (Manning & Woody, 1987), which complicates such estimations. For instance, the positive CD band near 200 nm, arising from β-structure, might vary in intensity by more than an order of magnitude in structurally similar proteins and in addition, the band position can change. Contributions from aromatic side chains, Trp residues in particular, are considered to be an important source of these variations in the far-UV region.

Despite both the wealth of information available on protein structures and the increasing use of CD, the contribution of the aromatic side chains to the CD spectrum has not yet been fully resolved. The sign, the magnitude, and the wavelength are aspects of the aromatic CD bands that are poorly understood. It is therefore essential to systematically investigate how aromatic side chains influence the appearance of a CD spectrum.

An empirical approach to allow estimations of the effects of tryptophan side chains on the far UV-CD spectrum is to use site-directed mutagenesis and replace the Trp residues of a protein one by one.

After assignments of various CD bands to specific Trp residues, these bands can be used to follow unfolding and refolding reactions, enabling specific characterization of folding intermediates, both kinetically and at equilibrium.

This approach has recently been used on the small single-domain proteins interleukin IL-1β (Craig et al., 1989) and barnase (Vuilleumier et al., 1993). In addition, aromatic residues have been analyzed theoretically (Manning et al., 1988; Manning & Woody, 1989), although no Trp residues have thus far been included in the calculations, because of particular difficulties due to the low symmetry of the indole ring (Woody, 1978).

In the present study, we applied the site-directed mutagenesis approach to the enzyme human carbonic anhydrase II (HCAII). The stability and folding properties of this protein have previously been studied in our laboratory (Freskgård et al., 1991; Mårtensson et al., 1992, 1993). HCAII is a zinc-containing, monomeric protein with a molecular weight of 29 300. The structure of this enzyme has been determined to a resolution of 1.54 Å by X-ray crystallography (Håkansson et al., 1992). HCAII has no disulfide bonds, and it is composed of a single domain. The main part of the

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[1] Abbreviations: CD, circular dichroism; HCAII, human carbonic anhydrase II; Trp, tryptophan; GuHCl, guanidine hydrochloride; HCA_{pwt}, pseudo-wild-type of HCA.

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secondary structure is a dominating β-sheet that extends throughout the entire molecule; some α-helical conformation (7%; Kabsch & Sander, 1983) is also present in the structure. HCAII contains a large fraction of Trp residues (7 Trp, 2.7%). An average protein has a Trp content of 1.7% (Singh & Thornton, 1992), which suggests that the Trp residues in HCAII may make important contributions to both the near- and far-UV CD spectra. Some of the Trp residues are located in two pronounced aromatic clusters in HCAII. Since exiton coupling between aromatic groups increases when they are in close proximity, perturbations of the CD spectra have probably obscured previous attempts to correctly estimate the amounts of the various types of secondary structure in HCAII. These problems might also be emphasized in this enzyme because of its relatively low contents of α-helix. Together these characteristics make this protein a good model for studying contributions to the CD spectrum made by structural elements other than secondary structure.

The aim of our study was to determine how the seven Trp residues influence the CD spectra of HCAII, both in the near- and the far-UV regions. The first crucial step toward this goal was to construct Trp mutants of HCAII in which one of the Trp residues at a time was lacking in the structure. It is very important that these mutants are sufficiently stable and possess a high enzymatic activity in order to ensure that the native conformation of the three-dimensional structure has not been changed. The magnitude and location of the contribution of each specific Trp residue to the CD spectrum can then be investigated, as well as the involvement of the immediate structural environment of the immobilized Trp residues in the native conformation.

Overall, these studies provide evidence that the Trp residues make significant contributions not only to the near-UV spectrum but also to the far-UV spectrum of HCAII.

MATERIALS AND METHODS

Chemicals. Guanidine hydrochloride (GuHCl, sequential grade) was purchased from Pierce and was made metal-free by extraction with dithizone (7 mg/L) in carbon tetrachloride. GuHCl concentrations were confirmed by measuring the index of refraction (Nozaki, 1972). All other chemicals were of biochemical or analytical grade.

Mutagenesis. The *in vitro* site-directed mutagenesis was performed essentially according to the method of Kunkel (1985), which is based on production of single-stranded DNA (ssDNA) in host cells that are deficient in the enzymes dUTPase (*dut*$^-$) and uracil *N*-glycosylase (*ung*$^-$). Mutants were obtained by in vitro second-stand synthesis from oligonucleotides containing mismatches, followed by transformation of *Escherichia coli* (*ung*$^+$, *dut*$^+$) and were identified by direct sequencing of the plasmid DNA. Usually, 50% or more of the clones contained the desired mutant plasmid. Uracil-containing ssDNA was obtained from the expression plasmid pACA (Nair et al., 1991), harbored in *E. coli* strain CJ236 (*ung*$^-$, *dut*$^-$) after infection with the helper phage M13K07. The plasmid pACA contains the gene for human carbonic anhydrase II, and this gene is under regulatory control of T7 RNA polymerase promoter; the plasmid also exhibits an F1 origin replication (which allows production of ssDNA) and a gene for ampicillin resistance. W192F was the only mutant that could not be isolated by the described procedure, and it was therefore constructed by using a method based on the polymerase chain reaction (PCR), as described by Hemsley et al. (1989). This work was done on a Perkin Elmer Cetus thermal cycler.

A plasmid containing the mutation was used to transfect *E. coli* BL21(DE3) (Studier & Moffat, 1986). The mutation and the complete coding region were checked by plasmid sequencing before the clone was used for protein production; this was done to ensure that no spontaneous mutations had occurred. All sequencing was performed according to the chain termination method (Sanger et al., 1977).

The following mutants were used in this study: W5F, W16F, W97C, W123C, W192F, W209F, and W245C. In all of these mutants, the single cysteine (206) was replaced by a serine to achieve a cysteine-free vector.

Protein Isolation and Purification. E. coli BL21(DE3) harboring the mutant plasmid was used for the production of the protein variants. The variants were purified by affinity chromatography (Khalifah et al., 1977), and the purity was verified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. For all the protein variants, a single band was obtained with no detectable impurity on the gel.

Circular Dichroism Measurements. Circular dichroism spectra were recorded on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France), employing constant N_2 flushing. The instrument was calibrated with an aqueous solution of *d*-10-(+)-camphorsulfonic acid at 290 nm. Each CD spectrum represents the average of three scans obtained by collecting data at 0.5 nm intervals with an integration time of 2 s. The three separate scans were inter compared before summation to detect possible alteration of the sample during the scan period. Each protein spectrum was corrected by subtraction of a spectrum obtained for a solution lacking the protein but otherwise identical. The resulting spectra were smoothed using the minimum filter included in the CD6 software. The ellipticity is reported as mean residue molar ellipticity ([θ], deg cm^2 dmol^{-1}) and calculated from

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

where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight (molecular weight 29 300 and 259 amino acid residues), c is the protein concentration (in g/mL, and l is the optical path length of the cell (in cm). Far-UV CD spectra were obtained by scanning the proteins in 1 mM sodium phosphate buffer, pH 7.5, in a 0.5-mm quartz cell; near-UV CD spectra were recorded using the same buffer in a 5-mm quartz cell. For the denatured protein in 5 M GuHCl, a 0.1 M Tris−H_2SO_4 buffer, pH 7.5, was used to maintain the correct pH. All spectra were recorded at 23 °C. The protein samples were dialyzed with three changes against the same buffer as used in the CD measurements. All solutions were filtered (Millipore; pore size: 0.4 μm) prior to use. The extinction coefficients were determined according to the method of Gill and von Hippel (1989). The protein concentration was 17 μM in all experiments in the near- and far-UV regions, and this was determined, after dialysis and filtration, with a Perkin-Elmer Lambda 5 spectrophotometer.

Structure Analysis. The crystallographic structure of HCAII was examined by using a Silicon Graphics workstation (IRIS INDOGO XS24), and the program INSIGHT II

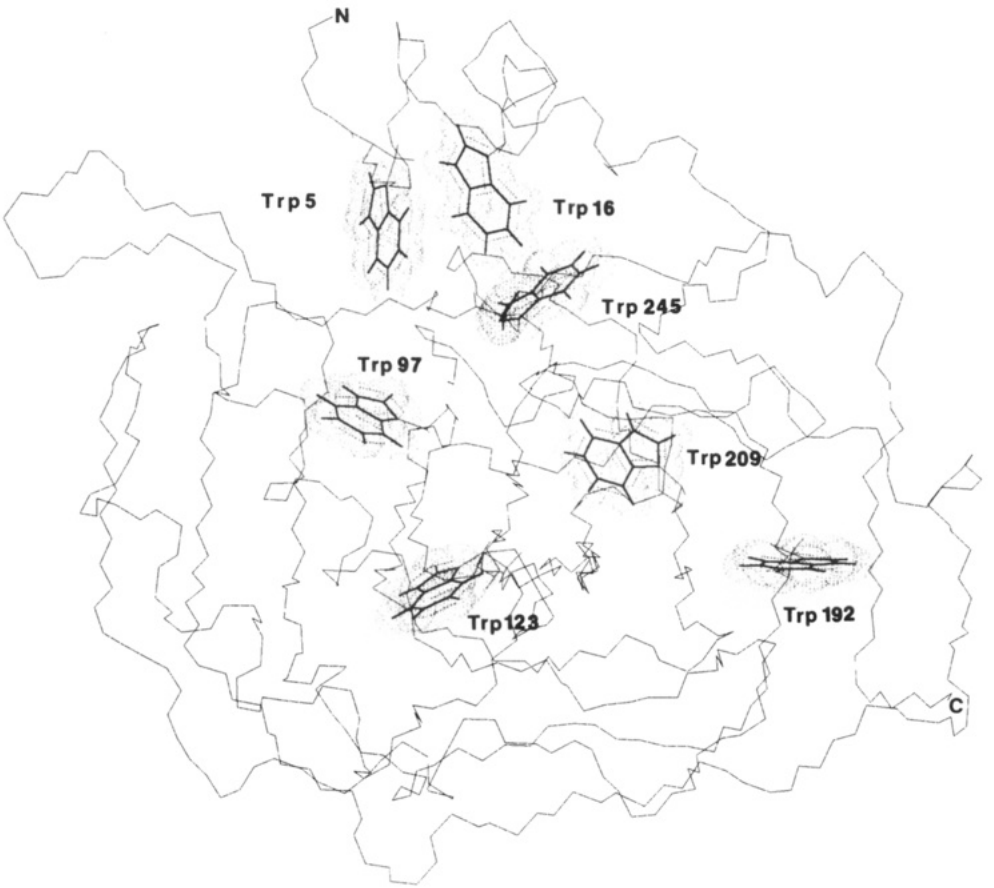

FIGURE 1: Polypeptide backbone of HCAII including the side chains of the seven Trp residues [from coordinates kindly provided by Håkansson et al. (1992)].

(Biosym, Inc). The coordinates were kindly provided by Håkansson et al. (1992).

RESULTS AND DISCUSSION

All the Trp mutants were constructed from a pseudo-wild-type form of HCAII (designated $HCAII_{pwt}$), in which the single cysteine at position 206 in the native enzyme had been replaced by a serine (C206S). Since $HCAII_{pwt}$ is devoid of cysteine residues, there can be no formation of intramolecular disulfide bridges at the site where a Trp residue in some of the mutants was replaced by a cysteine residue. This also eliminates the possibility of appearance of CD bands in the far-UV-region originating from intramolecular disulfide bonds. In some of the mutants, the Trp residue was replaced by a phenylalanine residue in order to minimize the destabilization of the native state as a consequence of the substitution. The unfolding and refolding behavior and the functional properties exhibited by $HCAII_{pwt}$ are practically indistinguishable from those of the wild-type enzyme (Mårtensson et al., 1993).

Before considering the significance of our CD results, it is first necessary to discuss a general problem, when dealing with alterations of the protein structure as here with the engineered Trp residues. In principle, substitution of a specific Trp residue might influence not only the point of mutation but also other parts of the protein structure, and if this is the case, such substitution might also alter the native environment of the remaining Trp residues. However, it is not likely that this occurs in the mutants used in this study, since these protein variants exhibit substantial enzyme activity. Among the mutants, W209F has the lowest activity, about 34% of that of the $HCAII_{pwt}$. The side chain of Trp209 is part of the active site, with its closest atoms only 5.3 Å from the Zn^{2+} ion, suggesting that the loss in activity is probably due to the mutation *per se*. Fulfillment of the activity criterion by the Trp mutants that well is a strong indication that the specific native conformation is formed and maintained.

Affinity chromatography was used to purify all the mutants ensuring that only active enzyme structures were selected for measurements. We have previously shown that a destabilized non-aromatic residue mutant of $HCAII_{pwt}$ (S29A; Mårtensson et al., 1992) has a near-UV CD spectrum that is essentially superimposible with that of $HCAII_{pwt}$. This shows that there is no direct correlation between a general destabilization of the native structure and conformational changes. In each mutant one Trp residue was replaced by a smaller amino acid residue, facilitating for the immediately surrounding structure to maintain the native conformation. Another strong indication that conformational alteration is restricted to the local area around the mutation point is that summation of the individual Trp spectra in the near-UV region yielded a spectrum that approximately reconstituted the native CD spectrum (see below and Figure 5).

The locations of the seven Trp residues, all of which were replaced in our study, are displayed in Figure 1. Trp5 and Trp16 are found in the N-terminus and are located in one of the two aromatic clusters present in the enzyme molecule. Trp residues 97, 123, 192, 209, and 245 are located in the central β-sheet region, which is a structural feature that

Table 1: Solvent Exposure, Conformation, and Inclusion of Tryptophan Residues in Aromatic Clusters[a]

Trp residue	solvent exposure (Å^2)[b]	conformation[c]	included in aromatic cluster[d]
5	22	extendend	yes
16	4	3_{10}-helix	yes
97	0	β-sheet	yes
123	6	β-sheet	no
192	13	β-sheet	no
209	4	β-sheet	no
245	47	extendend	no

[a] Refined structure of HCAII at 2.0 Å (Eriksson et al., 1988). [b] Solvent exposure calculation was performed according to Kabsch and Sander (1983). [c] Conformation defined according to Eriksson et al. (1988). [d] Whether or not included in an aromatic cluster in HCAII (Eriksson et al., 1988).

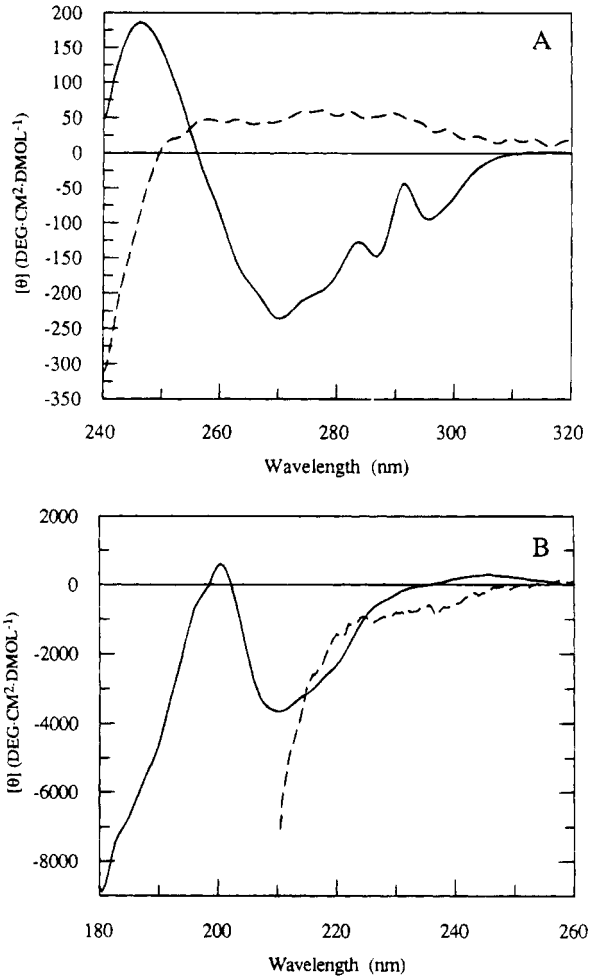

FIGURE 2: A: Near-UV CD spectrum of $HCAII_{pwt}$ in 0 M GuHCl (—) and 5 M GuHCl (- - -). B: The far-UV CD spectrum of $HCAII_{pwt}$ in 0 M GuHCl (—) and 5 M GuHCl (- - -).

dominates the entire protein molecule. Trp97 is completely buried and is part of the other major aromatic cluster in HCAII (Eriksson et al., 1988). The locations and exposure-to-solvent characteristics of the various Trp residues are given in Table 1.

CD Spectra of Native HCAII. An examination of the CD spectrum of $HCAII_{pwt}$ in the native state (Figure 2a) shows that it is indistinguishable from the previously reported spectrum (in the wavelength region 220−300 nm) of the wild-type enzyme (Beychock et al., 1966). With the development of CD spectropolarimeters, it became possible

Table 2: Close Contacts between Aromatic Residues and Trp Residues in HCAII[a]

Trp residue	Trp atom[b]	close residue[c]	distance (Å)[d]
5	ϵ1	His4(ϵ2)	4.1
	ζ3	Tyr7(η)	3.5
	ϵ3	Trp16(ζ2)	3.7
	δ1	Phe20(ϵ2)	3.8
	η2	His64(δ1)	4.3
16	ζ2	Trp5(ϵ3)	3.7
	ζ2	Tyr7(ϵ2)	3.8
	δ1	His15(β)	5.4
	γ	Phe20(δ2)	4.9
97	ζ3	Phe66(ϵ2)	4.4
	ϵ3	Phe95(γ)	5.0
	ϵ3	His96(γ)	6.2
	ζ3	Phe226(δ2)	3.9
123	δ1	Phe131(δ2)	3.9
192	ζ3	Phe147(ϵ2)	3.7
	ϵ1	Phe260(ζ)	4.5
209	ϵ3	His107(ϵ1)	3.6
	η2	His119(ϵ1)	3.5
	γ	Tyr194(ϵ2)	3.8
245	γ	Tyr7(δ2)	6.2
	γ	His96(γ)	6.2

[a] Refined structure of HCAII at 2 Å (Eriksson et al., 1988). Atoms at a distance of 8 Å or closer from the δ2 atom in the Trp residue were included. [b] Closest atom in the Trp residue. [c] Neighboring residue with the closest atom within parentheses. [d] Distances between the two defined atoms.

to extend these measurements to 180 nm. A comparison of the far-UV region (180−220 nm) of the CD spectra of HCA_{pwt} and the wild-type enzyme showed that the two enzyme forms were also identical in this spectral range (data not shown). The CD spectrum contains typical bands in the near-UV region, but this part of the spectrum is unusually complex and rich in details with multiple Cotton effects. The complexity of the near-UV spectrum most probably arises from exiton splitting of transitions from aromatic residues, mainly tryptophans. The exiton couplings observed probably arise due to the highly asymmetrical environment of the aromatic residues in HCAII, in which three of the Trp residues (nos. 5, 16, and 97) are also located in two aromatic clusters (Eriksson, 1988). The nearest neighboring aromatic amino acid residues to the various Trp residues in the enzyme (within 8 Å) are collected in Table 2. It is probably among these groups where exiton couplings occur that are responsible for the spectral complexity in the near-UV range. Notably, Trp residues 5 and 16 are close neighbors in the native structure. Hence, their CD spectra very likely are influenced of exiton couplings between the two. Therefore, we may not expect that their contributions to the total CD spectrum are additive.

Previously, description of the CD spectrum of HCAII in the near-UV region required the assumption of at least two major CD bands (at 249 and 270 nm) and at least two small bands (L_b transitions) to account for the fine structure above 280 nm (Beychock, 1966). According to our $HCAII_{pwt}$ CD spectrum (Figure 2a), there appears to be an additional band with a negative ellipticity at 278 nm.

The spectrum in the far-UV region has a comparatively low intensity (Figure 2b), which can be expected from a protein with a low α-helical content (7%; Kabsch & Sander, 1983). There is a negative band at 180 nm ($[\theta]$ = −8910 deg cm^2 dmol^{-1}), a positive band at 200 nm ($[\theta]$= 550 deg cm^2 dmol^{-1}) and a negative band at 210 nm ($[\theta]$= −3660

deg cm^2 dmol^{-1}). According to Manning and Woody (1987), the positions of these bands are in accordance with a β-sheet structure. In addition, the intensities of the bands are most likely affected by both positive and negative contributions from other secondary structures, as well as contribution from aromatic residues (see below). The comparatively low intensity of the CD band at 200 nm might indicate a twisted β-structure (Manning & Woody, 1987) that is in agreement with the crystal structure of HCAII, which shows that the protein contains a markedly twisted β-structure, consisting of 10 β-strands that are right-hand twisted about 220°.

CD Spectra of Denatured HCAII. The complex band pattern observed in the near-UV CD spectrum of the native protein has vanished in the corresponding spectrum of the GuHCl-denatured HCAII$_{pwt}$ (Figure 2a). This indicates that the asymmetric environment around the various aromatic amino acid residues in the native state was disrupted upon unfolding, resulting in a CD spectrum typical for an unordered polypeptide chain. Note that the CD spectrum does not, however, coincide with the baseline, as was previously reported (Beychock et al. 1966, Carlsson et al., 1973). Older CD instruments were probably not sensitive enough to resolve the CD spectrum from the baseline when GuHCl was present in the sample.

In the far-UV region, there is an increase in magnitude of the negative band at 210 nm (Figure 2b), which coincides with one of the minima in the native CD spectrum. CD spectra of unordered polypeptides are known to have negative ellipticities in the vicinity of 200 nm. For the denatured enzyme, recording spectra lower than 210 nm was prevented by the high absorbance of the GuHCl solution. As can be seen, denaturation does not substantially alter the CD spectrum in the wavelength region (215−225 nm) where changes in the contents of secondary structure are usually monitored. The reason for this small difference is due to the low α-helix content (7%) in the protein and is in addition due to the low ellipticity caused by β-structure (28% β-sheet in HCAII). In this wavelength region, the relatively small difference in the CD spectrum of the native and the denatured enzyme will, of course, make it difficult to use CD measurements in kinetic experiments to follow formation or breakage of secondary structure during refolding and unfolding reactions, respectively.

Contributions of Individual Tryptophans to CD Spectrum. (A) Near UV. As a first step in the assignment of the contribution of the Trp residues to the native CD spectrum, we recorded spectra in the near-UV region for all of the Trp mutants (Figure 3). In this range, inserted phenylalanine residues should not contribute to the CD spectrum. All of the Trp residues contribute to the CD spectrum over essentially the entire near-UV spectral range. The contribution of the deleted Trp residue can be obtained by subtracting the Trp mutant spectra from the HCAII$_{pwt}$ spectra. The results from such calculations are shown in Figure 4, and the wavelength extrema with accompanying ellipticities of the CD spectra of the individual Trp residues are compiled in Table 3. There are two Trp residues that make the largest contribution to the near-UV CD spectrum, namely, Trp97 and Trp245. The fine structure seen above 280 nm in the HCAII CD spectrum, with negative extrema at 287 and 296 nm, can also be detected to various extents in the spectra of the individual Trp residues (Figure 4). However, the magnitudes of these bands are dominating in the spectra of

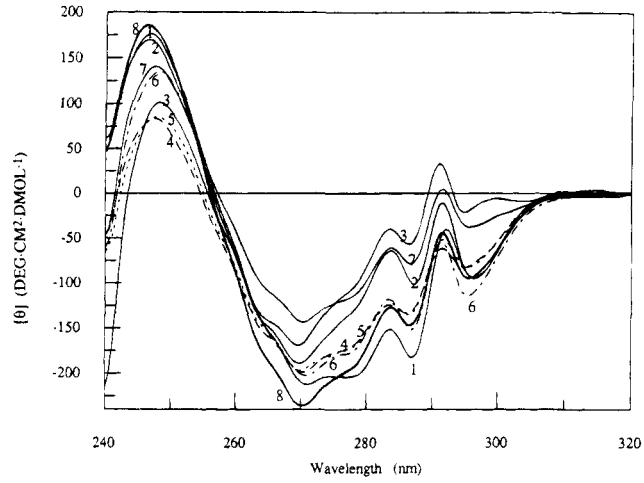

FIGURE 3: Near-UV CD spectra of Trp mutants of HCAII$_{pwt}$. Mutant with accompanying number and curve line: W5F:1 (—); W16F:2 (—); W97C:3 (—); W123C:4 (- - -); W192F:5 (•••); W209F:6 (— • —); W245C:7 (—); and HCAII$_{pwt}$:8 (—).

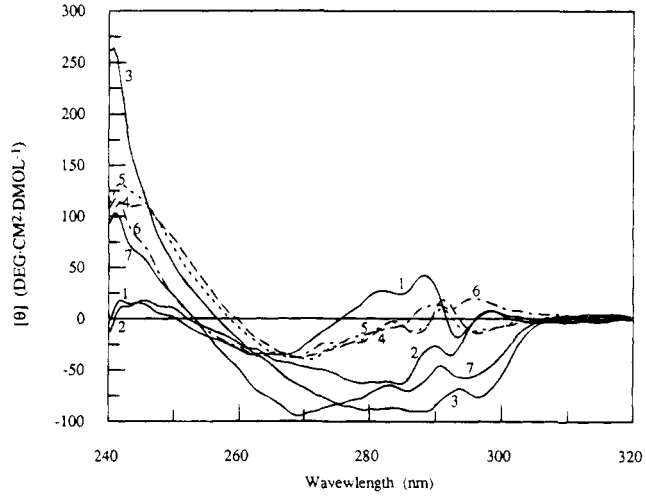

FIGURE 4: Contribution of individual Trp residues to the near-UV CD spectrum of HCAII$_{pwt}$. These spectra were obtained as difference spectra: HCAII$_{pwt}$ − Trp mutant. Tryptophan with accompanying number and curve line: W5:1 (—); W16:2 (—); W97:3 (—); W123:4 (- - -); W192:5 (•••); W209:6 (— • —); W245:7 (—).

Trp97 and Trp245, which are primarily responsible for the detected 287- and 296-nm-bands (L_b transition) in the native CD spectrum. Recently, we have shown that these residues are also the major Trp fluorescence emitters (Mårtensson et al., 1994).

One of the Trp residues (no. 123) does not have several but only one vicinal aromatic amino acid residue to interact with (Table 2), and interestingly Trp123 is the Trp residue that contributes least to the CD spectrum of HCAII$_{pwt}$ (Figures 4 and 7).

To obtain the CD spectrum made up from all the Trp residues in the near-UV region, the CD difference spectra of all the Trp mutants were added to obtain the total Trp CD spectrum of HCAII$_{pwt}$. This composite spectrum is exhibiting all features of the HCAII$_{pwt}$ spectrum, even in the fine structure above 280 nm (Figure 5). This clearly shows that Trp residues of HCAII are responsible for the main part of the CD spectrum in the near-UV region. Trp residues have also been claimed to be the major determinant of the near-UV CD spectrum (Strickland, 1974). The close qualita-

Table 3: Wavelength Extrema and Intensity Exhibited by Individual Trp Residues in Near-UV Region[a]

Trp residue	wavelength extremum[b]	molar ellipticity	
		θ[c]	$(\Delta\epsilon)$[d]
5	266	−8580	(−2.6)
	288	9880	(3.0)
	293	−3640	(−1.1)
16	280	−16900	(−5.1)
	292	−8580	(−2.6)
97	281	−23660	(−7.2)
	289	−24940	(−7.6)
	297	−20020	(−6.1)
123	270	−9620	(−2.9)
	287	−4160	(−1.3)
192	271	−10660	(−3.2)
	288	−3380	(−1.0)
209	269	−9360	(−2.8)
245	269	−24180	(−7.3)
	287	−17680	(−5.3)
	295	−14820	(−4.5)

[a] Obtained from difference spectra (pseudo-wild-type Trp mutant). [b] Detected extremum (see Figure 4). [c] Molar ellipticity per mole of protein. [d] Absorption coefficient per mole of protein.

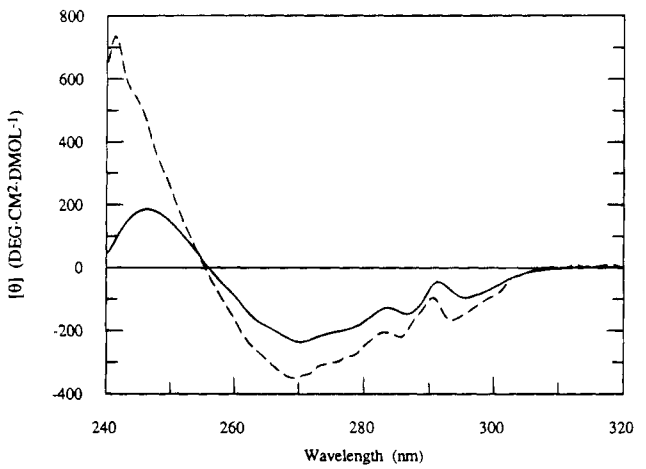

FIGURE 5: Comparison of the CD spectrum obtained after summation of the individual Trp CD spectra in the near-UV region from Figure 4 with the $HCAII_{pwt}$ CD spectrum. The summed Trp-CD spectra (- - -); $HCAII_{pwt}$ (—).

tive resemblance of this composite Trp-CD spectrum with the CD spectrum of $HCAII_{pwt}$ also strongly indicates that there is no major conformational change due to the Trp mutations, or more specifically, there are no major structural disturbances in the surroundings of the remaining Trp residues from the position where the mutation is made in the various Trp mutants. This is in spite of the fact that Trp5 and Trp16 are close to each other in the protein structure (Table 2). The differences of the magnitudes of the various CD bands in the wavelength region 255−320 nm from the composite and the $HCAII_{pwt}$ spectra are in addition no greater than can be explained by some uncertainties in the protein concentration determinations of the different Trp mutants which were done from extinction coefficients calculated according to Gill and von Hippel (1989).

The individual Trp CD spectra, obtained by subtraction, also probe the local environment; this can for instance be seen for Trp245, whose near CD spectrum is blue-shifted relative to those of the other Trp residues (Figure 4). X-ray structure analysis revealed that Trp245 is the most solvent accessible of the Trp residues, which agrees with the observed CD spectrum (Table 1). Considering crystal structure, Trp97 is the Trp residue that is buried to the greatest extent; this is also reflected in the CD spectrum of Trp97, which is significantly red-shifted (Figure 4). The usefulness of the Trp residues as spectroscopic probes of the properties of the surroundings in solution has also been corroborated by recent fluorescence measurements on these Trp mutants. In line with the observed shifts of the CD spectra and location in the crystal structure, the fluorescence spectra of Trp245 and Trp97 exhibited marked red- and blue-shifts, respectively (Mårtensson et al., 1994).

Strikingly, in the near UV-region, all of the individual tryptophan CD bands corresponding to L_a transitions (approximately 250−280 nm) are of negative sign, although previous theoretical calculations indicate a preference for positive contributions from Trp residues (Woody, 1978). Previously, a negative contribution in this wavelength region has experimentally been shown only for one of the three Trp residues in barnase (Vuilleumier et al., 1993), while the single Trp residue in interleukin IL-1β has a corresponding positive ellipticity (Craig et al., 1989). In $HCAII_{pwt}$, however, the absolute ellipticities of this transition from the Trp residues (Table 3) are of a magnitude similar to that seen in barnase and interleukin IL-1β.

Several proteins have strong positive CD bands in the 225−230 nm region, probably attributable to aromatic side chains. Still other proteins, among them HCAII, exhibit positive bands in the 230−250 nm region, which in a few cases have been assigned to aromatic transitions with a red-shift of the apparent maximum due to strong negative contributions of the peptide groups at shorter wavelengths (Woody, 1978). Figure 4 verifies this situation for HCAII, since all individual Trp residues contribute positively in this wavelength region. The total contribution of the summed individual Trp spectral bands exceeds here that of the $HCAII_{pwt}$ ($[\theta] = 738$ and 186 deg cm^2 dmol^{-1}, respectively), indicating that other aromatic chromophores, most likely tyrosine side chains, also contribute to the spectrum. In the native state of HCAII these tyrosines then probably contribute negatively to the ellipticity in this wavelength range. As indicated earlier, this CD band may also contain contributions from interactions between the closely interacting Trp5 and Trp16. When a Trp residue is removed such an extra interaction is lost, and the contribution from such an interaction will be summed twice when the composite Trp spectrum is calculated also giving rise to different magnitudes of this CD band.

(B) Far UV. There are also significant changes in the far-UV region of the CD spectra of $HCAII_{pwt}$, when a Trp residue is replaced (Figure 6). The magnitudes of the CD bands attributed to each of the individual Trp vary between maximal values of +2500 and −1500 deg cm^2 dmol^{-1} at different wavelengths. Thus, for a low intensity spectrum, like that of HCAII, each Trp contributes a sizeable fraction to the spectrum. In a case where Trp residues contribute similarly, the additive effect of the Trp spectra can then make significant changes of a CD spectrum. The difference spectra between the spectrum of $HCAII_{pwt}$ and those of the various Trp mutants are shown in Figure 7 and illustrate the apparent spectral contribution of each Trp in the far-UV region. For the phenylalanine-substituted mutants, putative contributions from the inserted phenylalanine residues are of course included in the corresponding Trp CD spectrum.

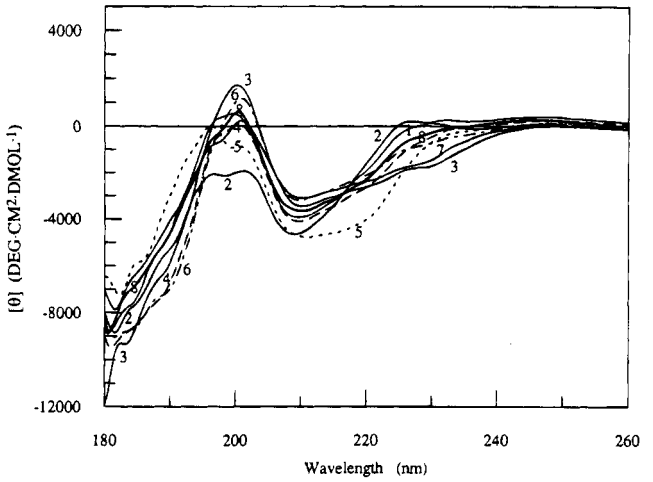

FIGURE 6: Far-UV CD spectra of the Trp mutants of $HCAII_{pwt}$. Mutant with accompanying number and curve line: W5F:1 (—) ; W16F:2 (—); W97C:3 (—); W123C:4 (- - -); W192F:5 (•••); W209F:6 (— • —); W245C:7 (—); $HCAII_{pwt}$:8 (—).

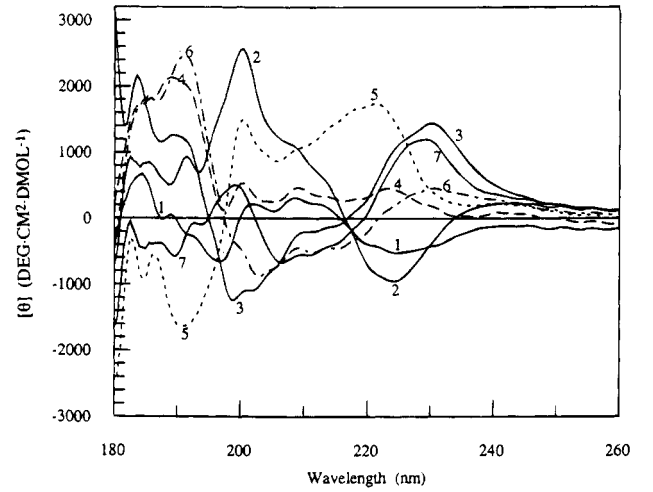

FIGURE 7: Contribution of individual Trp residues to the far-UV CD spectrum of $HCAII_{pwt}$. These spectra were obtained as difference spectra: $HCAII_{pwt}$ − Trp mutant. Tryptophan with accompanying number and curve line: W5:1 (—); W16:2 (—); W97:3 (—); W123:4 (- - -); W192:5 (•••); W209:6 (— • —); W245:7 (—).

In the wavelength region 220−230 nm, from which helix content is frequently estimated (mostly at 222 nm), all of the Trp residues contribute substantially to the CD spectrum. Only two of the Trp residues (nos. 5 and 16) cause negative ellipticity here. These residues are located in the N-terminus, where Trp16 is included in a 3_{10}-helical segment, indicating that the negative contributions might arise from coupling with the transitions of the aromatic side chains and the helical peptide $\pi-\pi^*$ transitions. It has also been shown for helical model peptides that Trp residues induced negative aromatic CD bands in this wavelength region (Chakrabartty et al., 1993). The rest of the Trp residues, all positioned in various β-strands, also contribute significantly, but positively in this wavelength region (220−230 nm). Apparently Trp residues located in different secondary structural contexts can contribute both negatively and positively here, obscuring α-helix calculations from measurements in this wavelength region. At 222 nm, where α-helix formation usually is monitored, Trp192 gives the largest contribution.

When the analysis is extended down to 180 nm, it is obvious that contributions from Trp residues also are considerable in this part of the CD spectrum (Figure 7). This means that Trp residues will interfere with predictions of the contents of various types of secondary structure based on algorithms using ellipticity data in the wavelength region 180−260 nm.

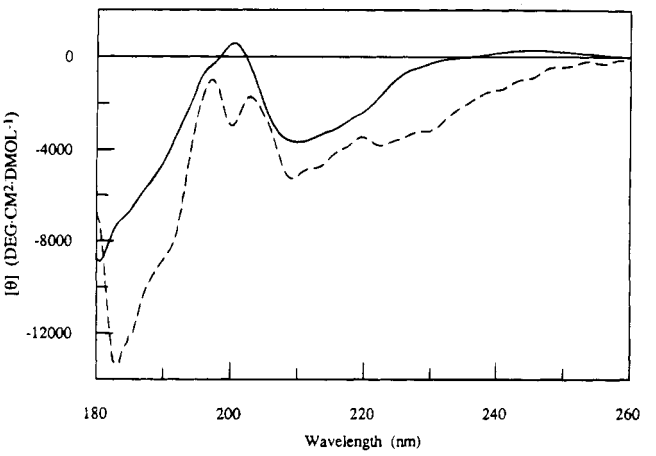

FIGURE 8: Far-UV CD spectrum of a hypothetical Trp-less $HCAII_{pwt}$. This spectrum was obtained after subtraction of the spectral contributions of all the individual Trp residues from the CD spectrum of $HCAII_{pwt}$. Trp-less $HCAII_{pwt}$ (- - -); $HCAII_{pwt}$ (—).

A hypothetical $HCAII_{pwt}$ CD spectrum free from Trp contributions is obtained by subtracting all Trp contributions from the $HCAII_{pwt}$ spectrum. As seen in Figure 8, the Trp-free spectrum is distinct from the $HCAII_{pwt}$ spectrum.

CONCLUSIONS

By inspecting the CD spectra of the individual tryptophans (Figure 4), it is possible to find wavelengths that can be used specifically for monitoring the refolding process in the substructure surrounding some of the Trp residues. In this way the replaced Trp residues can serve as spectroscopic probes to register the formation of an asymmetric environment, both kinetically and at equilibrium. Trp97 and Trp245 are, for instance, the only Trp residues that give rise to any significant CD bands in the near-UV region of 295−300 nm. Thus, changes in ellipticities in that wavelength region can be employed to follow the formation of tertiary interactions. This can be done specifically in the substructures containing Trp97 and Trp245 by studying the W245C and W97C mutants, respectively, in regard to reappearance of ellipticity during the refolding process.

As we have demonstrated, there is a considerable interference of CD bands from the Trp residues in the far-UV region spectrum of $HCAII_{pwt}$ (Figure 7) making it difficult to assign changes in ellipticities to changes in secondary structure content. However, if the spectrum of W192 is excluded from the CD spectra of the individual Trp residues in this wavelength region, an approximate isosbestic point is exhibited at 218 nm with essentially no ellipticity. By using the W192F mutant, contributions made by the Trp residues in kinetic measurements of secondary structure formation at this wavelength can be eliminated.

Finally, the absolute magnitudes of the CD bands of the Trp residues are in general terms relatively small (*i.e.*, these contributions will have a minor effect on CD spectra for proteins with substantial amount of α-helix structure), but for proteins possessing a CD spectrum of low intensity, Trp residue CD bands can markedly influence the far-UV

spectrum, especially in proteins containing several Trp residues.

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