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Circular Dichroism of Cyclic Hexapeptides with One and Two Side Chains*

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ABSTRACT: We report ultraviolet circular dichroism curves for several cyclic hexapeptides of glycine, leucine, tyrosine, and histidine in neutral aqueous solution. Our interpretation is assisted by previously reported nuclear magnetic resonance data on these compounds which indicate that they contain two transannular hydrogen bonds. These structures are fairly rigid, but have some conformational mobility. A circular dichroism band at 198 nm is assigned to the amide π–π^* band while bands at 214 and 222 nm are assigned to amide n–π^*.

A weak band seen in *cyclo*-Gly_5-Leu as well as in random coil polypeptides at 230 nm is interpreted as a residual of overlapping n–π bands and not as a result of an absorption band at that wavelength. Circular dichroism bands due to the neutral tyrosyl side chain are identified at 275, 228, and 198 nm and a band due to histidyl side-chain absorption is identified at 211 nm. The amide backbone perturbs the symmetry of the side chain leading to Cotton effects at the wavelengths of side-chain absorptions. In addition, we also observe the effect of the side chain in modifying the optical activity of both the n–π^* and π–π^* transitions of the amide. We propose that the conformation of *cyclo*-Gly_5-Leu is an equilibrium between two or more forms, one of which is preferred in *cyclo*-$(Gly_2\text{-Leu})_2$. The similarity of the circular dichroism curve of the former compound to that of polyglutamic acid in neutral solution leads us to suggest that a similar combination of conformations could contribute in random coil systems.

The optical activity curves for model peptides are often used in the interpretation of protein optical activity spectra. The model structures most commonly used are the α helix, random coil, and more recently the anti-parallel β form (Greenfield *et al.*, 1967). Although these models provide a general framework for the interpretation of protein optical rotatory dispersion and circular dichroism, it is clear that globular proteins contain rigid conformations other than α helix and β forms and that they are unlikely to contain any truly random coils. In the present study, we report circular dichroism curves for four cyclic hexapeptides of relatively rigid conformation for which we have structural information from nuclear magnetic resonance (Kopple *et al.*, 1969a,b). We have studied their optical activity in the hope of providing a better understanding of the contributions to optical activity of various rigid polypeptide structures other than the presently available models, α helix and β forms.

The nuclear magnetic resonance spectra of the cyclic

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hexapeptides, *cyclo*-Gly$_5$-L-Tyr (Kopple *et al.*, 1969a), *c*-Gly$_5$-L-Leu, and *c*-Gly$_2$-L-Tyr-Gly$_2$-L-His (Kopple *et al.*, 1965b), have recently been reported. These workers interpret their results as evidence for a conformation of the type originally proposed by Schwyzer and coworkers (Schwyzer *et al.*, 1958, 1964; Schwyzer, 1959). This structure contains two planar antiparallel extended peptide segments joined at their ends by peptide bonds perpendicular to the average ring plane, and containing two transannular hydrogen bonds. The reader is referred to Kopple *et al.* (1969b) for a further discussion of this model.

We feel that our results demonstrate the value of using nuclear magnetic resonance data on conformation of peptides to assist in the interpretation of optical activity curves. Nuclear magnetic resonance provides a general indication of the shape of the structure and specifies certain allowed conformations. The circular dichroism, since it is so sensitive to minor variations of structure, can be used to distinguish among various possible conformations consistent with the nuclear magnetic resonance. Madison and Schellman (1970) have recently illustrated the power of this approach in small peptides in a series of peptides containing proline.

We are dealing in some cases with side chains having chromophores which absorb in the near-ultraviolet region. Since separation of the various contributions to the optical activity is more straightforward in circular dichroism than it is in the optical rotatory dispersion, we have chosen the former method. Circular dichroism measurements offer the inherent advantage of displaying discrete bands near the electronic absorption bands of the molecule and are not complicated by the background tail due to strong optically active transitions below 200 nm. This tail, which occurs in the optical rotatory dispersion curve, tends to obscure weaker Cotton effects occurring at longer wavelengths such as those due to side chain chromophores.

The Cotton effects present in the circular dichroism spectrum of these cyclic hexapeptides occur in the ultraviolet between 180 and 300 nm. The electronic transitions include the amide $n-\pi^*$ whose wavelength deviates from the α-helix position 222 nm due to solvent effects. Also due to the amide is the strong $\pi-\pi^*$ transition at 190 nm which is split into components appearing at various wavelengths by degenerate or exciton interaction (Woody and Tinoco, 1967). We also deal with the chromophoric side chains histidine and tyrosine. There are absorption bands for histidine at 212 nm (ϵ 6000), and for tyrosine at 275 nm (ϵ 1200), 225 nm (ϵ 7800), and 192 nm (ϵ 47,000) (Wetlaufer, 1962). For the histidyl side chain, there is also the possibility that an azine $n-\pi^*$ transition could contribute Cotton effects; however, the contribution of such transitions to circular dichroism is not well understood.

Experimental Section

The cyclic and linear polypeptides investigated in this work were prepared and supplied to us by Dr. K. D. Kopple of this department. The cyclic hexapeptide *c*-(Gly$_2$-L-Leu)$_2$ was first reported by Schwyzer and Sieber (1958). The preparation of *c*-Gly$_5$-Leu, *c*-Gly$_5$-Tyr, and *c*-Gly$_2$-Tyr-Gly$_2$-His is described by Kopple *et al.* (1969a,b). Water solutions in the concentration range 10^{-4}–10^{-3} molar per average residue molecular weight were prepared for the circular dichroism measurements. The concentrations of the solutions used in the circular dichroism measurements were determined from the extinction coefficients at the maximum absorption near 190 nm. These coefficients were determined from the ultraviolet absorption spectrum of carefully weighed samples of these polypeptides taken on Cary 14 and 15 spectrophotometers. The extinction coefficients are given in Table I.

TABLE I: Molar Extinction Coefficients per Average Residue Molecular Weight for the Absorption Bands of Some Cyclic Polypeptides in Water Solutions.

Compound	λ Max (nm)	ϵ/av Residue Mol Wt
c-Gly$_5$-L-Leu	190	6,450
c-Gly$_5$-L-Tyr	192	11,900
	224	1,525
	275	220
c-Gly$_2$-L-His-Gly$_2$-L-Tyr	192	13,350
	222	2,230
c-(Gly$_2$-Leu)$_2$	188	6,840

Circular dichroism was measured on the Cary 60 spectropolarimeter with the Model 6002 circular dichroism accessory. The cells employed were of fused silica with path lengths varying from 0.1 to 5 cm. The resulting optical activities are reported here as the ellipticity per average residue molecular weight.

$$[\theta]_\lambda = \frac{\theta M}{10c'l}$$

θ is the ellipticity in degrees at a given wavelength, M is the average residue molecular weight, c' is the grams of solute per cubic centimeter of solution, and l is the path length in centimeters. The resulting units of the ellipticity per residue are deg cm^2 dmole^{-1}. It should be recognized that the ellipticity of all circular dichroism bands are thus given on a per amide basis. The side-chain Cotton effects should be multiplied by a factor of 6 to give ellipticity per chromophore.

Results

Absorption Spectra. The short-wavelength absorption arises mainly from the allowed amide $\pi-\pi^*$ transition. In the compounds containing histidine and tyrosine side chains, these chromophores also contribute to the absorption maxima near 190 nm. There is an indication of the conformational properties of the peptides *c*-Gly$_5$-Leu and *c*-(Gly$_2$-Leu)$_2$ in their relative absorption intensities. *c*-(Gly$_2$-Leu)$_2$ is expected to have a more rigid conformation and is hyperchromic with respect to *c*-Gly$_5$-Leu.

This hyperchromic effect has also been discussed in cyclic hexapeptides by Ovchinnikov *et al.* (1969). Similar trends were observed in their work, but it is difficult to compare our results since their data are reported at fixed wavelengths and the maxima are not indicated. The exciton coupling varies among these compounds and the absorption maxima may differ by several nm.

Circular Dichroism of c-Gly$_5$-Leu. From the circular dichroism curve of *c*-Gly$_5$-Leu in Figure 1, three distinct circular dichroism bands are recognizable. There is a very weak negative band centered about 230 nm with an ellipticity value of -50 ± 10. This band does not appear in α-helical

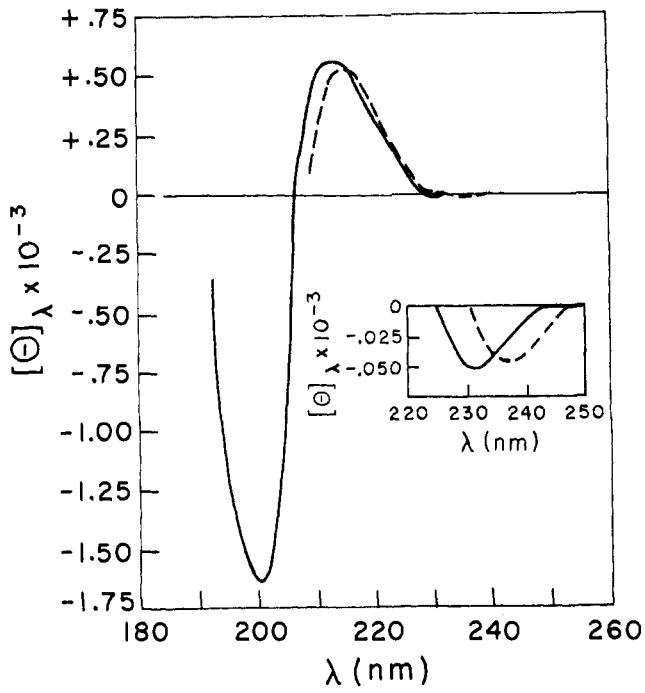

FIGURE 1: Circular dichroism of *c*-Gly_5-L-Leu: in water (——) and in methanol (-----).

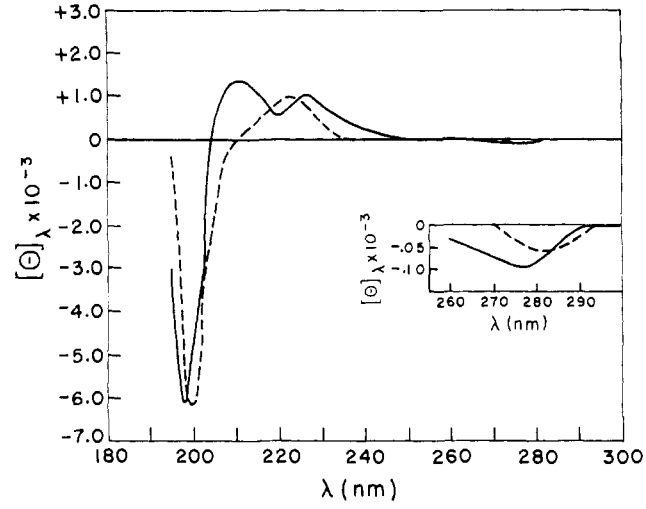

FIGURE 2: Circular dichroism of *c*-Gly_5-L-Tyr (——) and H-Gly_2-L-Tyr-Gly_3-OH (-----) in water.

or antiparallel β conformations of polypeptides, but seems to be a general phenomenon associated with polypeptides in the random coil conformation (Carver *et al.*, 1966). It is also reported in the poly-L-proline I conformation (Timasheff *et al.*, 1967). The weak positive band near 214 nm has an ellipticity value of 550 ± 50. The remaining circular dichroism band at 200 nm is negative with an ellipticity of −1650 ± 150. Both in sign and in wavelength, these latter bands are similar to those found for polypeptides in the random coil conformation (Carver *et al.*, 1966; Deutsche *et al.*, 1969). The magnitudes of ellipticity, however, are substantially smaller than those found for most random coil polypeptides. Random coil poly-L-glutamic acid shows an ellipticity of approximately −27,000 at 200 nm (Carver *et al.*, 1966).

In order to assist in determining the molecular electronic origin of the circular dichroism band of *c*-Gly_5-Leu, the circular dichroism curve was also measured in methanol where the conformation is presumably identical with that in water (Kopple *et al.*, 1969b). The methanol results (Figure 1) show a significant shift to longer wavelengths for both the 230-nm band and the 214-nm band. Such a shift is typical of an n–π^* transition.

Circular Dichroism of c-Gly_5-Tyr. The circular dichroism spectrum for the tyrosyl cyclic hexapeptide (Figure 2) is in some ways similar to that of the leucyl compound. In addition to the bands directly attributed to amide backbone bands, the tyrosyl derivative displays side-chain optical activity. The new circular dichroism bands at 275–278 and at 225 nm can be assigned to tyrosine absorption bands. The circular dichroism band at 198 nm is most likely comprised of both amide π–π^* and side-chain optically active absorption bands. The circular dichroism band at 210 nm corresponds to the amide band found in the leucyl derivative at 214 nm. The very weak band seen in the circular dichroism of *c*-Gly_5-Leu is obscured in *c*-Gly_5-Tyr due to strong overlapping by the positive band at 225 nm resulting from the tyrosyl side chain. In poly-L-tyrosine, there is also a band at 240 nm (Beychok and Fasman, 1964), apparently arising from an absorption band of the negatively ionized side chain. In our neutral solution, we do not detect a circular dichroism band at this wavelength.

The circular dichroism of the linear form of this peptide, H-Gly_2-Tyr-Gly_3-OH, is also included in Figure 2. The contribution of the amide chromophore to the optical activity should be nearly absent here, with most of the circular dichroism arising from tyrosyl side-chain absorption. Certainly the amide n–π^* Cotton effect seen at 210 nm in *c*-Gly_5-Tyr is not evident in the linear analog.

Circular Dichroism of c-(Gly_2-Leu)$_2$. The circular dichroism of *c*-(Gly_2-Leu)$_2$ in water is given in Figure 3. The nuclear magnetic resonance of this compound indicates that it has a structure similar to the previously reported cyclic hexapeptides we have dealt with in this study (K. D. Kopple, private communication, 1970). Although the chromophores and gross conformation are the same as those of *c*-Gly_5-Leu, the circular dichroism is quite different. Instead of a positive amide n–π^* band there is a negative one. The negative band in the π–π^* region is small relative to that in the random coil. The high absorption in this region makes it extremely difficult to make precise measurements. Hence, the molar ellipticity has considerable uncertainty in this case as well as in *c*-Gly_5-Leu.

Circular Dichroism of c-Gly_2-Tyr-Gly_2-His. The circular dichroism of *c*-Gly_2-Tyr-Gly_2-His (Figure 4) shows a large negative band at 198 nm. This band apparently results from the tyrosine chromophore and the allowed amide π–π^* transitions. The negative band at 217 nm will be shown to result from the amide n–π^* and partly from the histidyl side-chain absorption. We have carried out these measurements both in water solution and in 0.01 M phosphate buffer (pH 7.9) and obtained identical results. Neither side chain in this compound is appreciably ionized.

Discussion

Compounds Having only Amide Chromophores. Although the same type of structure with transannularly directed hydrogen bonds is deduced from the nuclear magnetic resonance (Kopple *et al.*, 1969b; K. D. Kopple, private communication, 1970), the comparison of the circular dichroism curves of *c*-Gly_5-Leu (Figure 1) and *c*-(Gly_2-Leu)$_2$

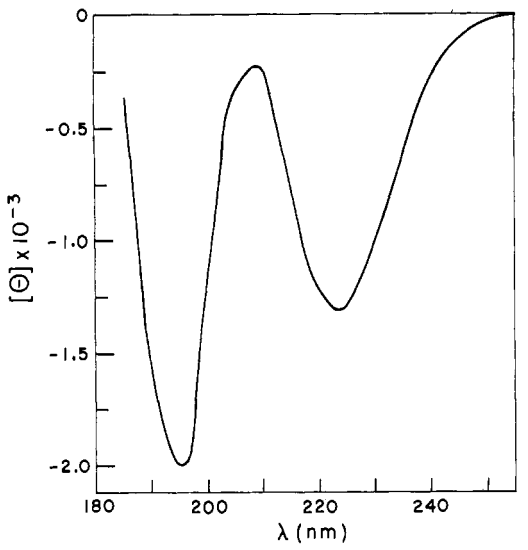

FIGURE 3: Circular dichroism of *c*-(Gly_2-L-Leu)$_2$ in water.

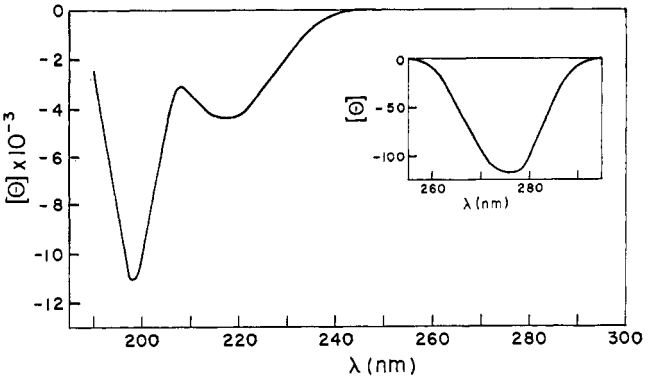

FIGURE 4: Circular dichroism of *c*-Gly_2-L-Tyr-Gly_2-L-His in water.

(Figure 3) indicates that these two peptides must have some conformational differences. Molecular models of *c*-Gly_5-L-Leu show that the transannularly hydrogen-bonded structure can exist in two distinct conformations depending upon whether the end amide oxygens are on the same side (C_2 conformation) or if they are on opposite sides (centrosymmetric conformation) of the average plane of the ring (Kopple *et al.*, 1969b). The latter conformation is illustrated in Figure 5. Opposite signs of ellipticity for these conformations might tend to cancel one another and the resulting weak optical activity for this cyclic hexapeptide could be explained by having a small excess of one of the two conformations.

A preliminary study of the rotational strength predicted for the n–π^* transition has been made using the calculations of Bayley *et al.* (1969). It does appear that the rotational strength of the two conformations is opposite. Moreover, the calculations indicate that it is the C_2 conformation which has the negative n–π^* Cotton effect. Inspection of space-filling models also indicates that the C_2 conformation is preferred when two side chains are present at opposite ends of the structure. We tentatively conclude that the difference in circular dichroism for these two compounds results from the preference of *c*-(Gly_2-Leu)$_2$ for the C_2 conformation and that the compound containing a single side chain has additional conformations which retain two transannular hydrogen bonds.

Any comparison for our curves for *c*-Gly_5-Leu to those of the random coil polyglutamic acid in neutral solution is complicated by the complex statistical nature of the random coil conformation. Myer (1969) has assigned the weak negative circular dichroism band at 238 nm in neutral polyglutamic acid to an overlapping of bands rather than to a distinct electronic transition at the wavelength. We find such a conclusion attractive since the amide does not have any known absorption band in the 230- to 238-nm region. Similarly, we conclude that the apparent circular dichroism band at 230 nm in *c*-Gly_5-Leu is not a true band assigned to a distinct amide absorption band at that wavelength. We interpret this band as the negative tail of an n–π^* band at shorter wavelength, as seen in *c*-(Gly_2-Leu)$_2$ (Figure 3). The apparent band at 230 nm in *c*-Gly_5-Leu is a result of cancelling positive (214 nm) and negative (222 nm) contributions. The differences in wavelength for the n–π^* bands probably result from slightly different solvation of the six amide oxygens. These differences in solvation might be suspected from the Schwyzer structure (Kopple *et al.*, 1969b), since two of the amide oxygens are hydrogen bonded across the peptide ring.

Some recent studies have found substantial variability in the circular dichroism of the random coil polypeptides and we may draw some interesting parallels between the data of Fasman *et al.* (1970) and our data of Figures 1 and 3. They report circular dichroism data for random coils in dried films which resemble our data on *c*-(Gly_2-Leu)$_2$ (Figure 3). They interpret the difference between dry films and neutral polyglutamic acid solutions as a result of freezing in of certain conformations in the dried films due to a loss of degrees of freedom in drying. Although we do not know what type of conformations contribute to the circular dichroism of neutral polyglutamic acid and which conformations are frozen out on forming dry films, it is possible that the conformations resemble those of *c*-Gly_5-Leu and *c*-(Gly_2-Leu)$_2$.

Effect of the Tyrosyl Side Chain. The circular dichroism band appearing at 210 nm in *c*-Gly_5-Tyr which we assign to the amide backbone, is approximately 2.8 times stronger in the tyrosyl-containing peptide than in the leucyl peptide.

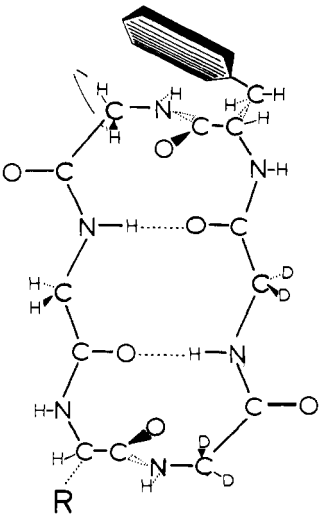

FIGURE 5: Conformation of cyclic hexapeptides bearing a benzyl side chain proposed from proton magnetic resonance data. C_2 symmetry is shown for the peptide backbone (Kopple *et al.*, 1969b, Figure 8).

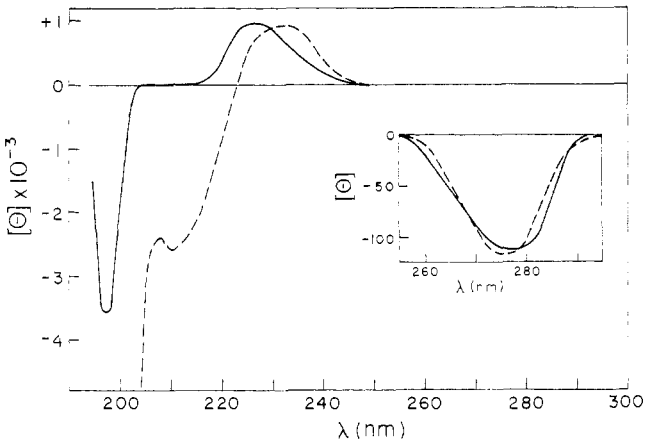

FIGURE 6: Difference curves showing the side chain Cotton effects: of tyrosine in *c*-Gly_5-Tyr (——), of tyrosine plus histidine in *c*-Gly_2-Tyr-Gly_2-His (-----). See text for method of calculation.

Neither the nuclear magnetic resonance results (Kopple *et al.*, 1969a,b) nor our inspection of space filling models lead us to expect any differences in peptide backbone configuration between *c*-Gly_5-Tyr and *c*-Gly_5-Leu. Most of the steric constraint comes from the β carbon atom which is common to both side chains. Therefore, we propose that the enhanced amide circular dichroism results from coupling between the tyrosyl and amide chromophores. This coupling also gives rise to the tyrosyl chromophore circular dichroism bands at 275 and 225 nm. Moreover, one would also expect that the amide contribution to the ellipticity of the 198-nm band in the tyrosyl compound to be different from that found for it in the leucyl derivative.

In order to separate the amide circular dichroism bands from the tyrosyl bands in *c*-Gly_5-Tyr, we will subtract the circular dichroism due to amide from the circular dichroism of *c*-Gly_5-Tyr. The circular dichroism due to amide is obtained from the circular dichroism of *c*-Gly_5-Leu, appropriately scaled to reflect the enhancement of the amide n–π^* band by the tyrosyl side chain. The factor, f, in eq 1 reflects the

$$[\theta]_{Tyr} = [\theta]_{c\text{-}Gly_5\text{-}Tyr} - f[\theta]_{c\text{-}Gly_5\text{-}Leu} \quad (1)$$

enhancement of the amide circular dichroism in *c*-Gly_5-Tyr as a result of coupling of the amide to the tyrosyl chromophore. This factor is chosen (f = 2.8) to give no circular dichroism band at 210 nm in the difference curve, [θ] Tyr. There is no absorption band at 210 nm in tyrosine. Since all these circular dichroism values are reported per amide residue, the tyrosine curve should be multiplied by six to yield the contribution per tyrosine.

The difference curve of Figure 6 may be compared to the circular dichroism of H-Gly_2-Tyr-Gly_3-OH (Figure 2). This open-chain peptide contains little amide contribution to the circular dichroism at 210 nm. The tyrosine contribution at 225 nm agrees reasonably well with our difference curve, while the 198-nm bands do not agree. Obviously, the coupling of the tyrosine chromophore to the π–π^* band of the amide is quite different from that to the n–π^* band.

The transition electric dipole of the strong absorption band in tyrosine interacts with the amide π–π^* transition dipole moment of a nearby amide by a coupled oscillator mechanism analogous to that in polynucleotides (Bush and Tinoco, 1967). This coupling leads to a double Cotton effect superimposed on the usual amide excition band. This latter band is seen in *c*-Gly_5-Leu (Figure 1) and in *c*-$(Gly_2\text{-}Leu)_2$ at 197–200 nm and is small in our compounds. The long wavelength branch of the coupled oscillator band for tyrosine is seen at 197 nm in *c*-Gly_5-Tyr (Figure 2) and in *c*-Gly_2-Tyr-Gly_2-His (Figure 4). Since such double Cotton effects conserve rotational strength, the other half of this double Cotton effect should be a positive band of similar magnitude paired with this band and having a maximum at 183–185 nm. Such a band is beyond the wavelength range of our experiment. The fact that the long-wavelength component of this double Cotton effect is negative implies that there is a left-handed helical relationship between the tyrosyl 192-nm transition dipole and that of the π–π^* in the amide to which the tyrosine is closest and hence most strongly coupled (Tinoco, 1963).

Two Chromophoric Side Chains --c-Gly_2-Tyr-Gly_2-His. Since most of the steric constraint in these systems results from the β-carbon atoms, we expect the allowed conformations of *c*-Gly_2-Tyr-Gly_2-His to be quite similar to those of *c*-$(Gly_2\text{-}Leu)_2$. We will interpret the differences in these two circular dichroism curves to be the result of the chromophores of the tyrosyl and histidyl side chains and their perturbation on the amide bands.

First let us take note of the similarity between the isolated 275-nm circular dichroism bands in *c*-Gly_5-Tyr and in *c*-Gly_2-Tyr-Gly_2-His. We interpret this as an indication of the similarity in the conformation of the tyrosyl side chains in the two compounds. Also there is no apparent interaction between the histidyl and tyrosyl side chains. This is to be expected from the conformation proposed in nuclear magnetic resonance studies (Kopple *et al.*, 1969b). These two side chains are at opposite ends of the structure.

In separating the contribution of the histidyl and tyrosyl chromophores from that of the amide backbone in *c*-Gly_2-His-Gly_2-Tyr, we will follow a procedure similar to that used in our treatment of *c*-Gly_5-Tyr. We use *c*-$(Gly_2\text{-}Leu)_2$ as a model for the amide contribution to the circular dichroism. Again, we must scale the amide contribution to reflect the enhancement of the amide n–π^* circular dichroism by coupling of the chromophoric side chains.

$$[\theta]_{His + Tyr} = [\theta]_{c\text{-}Gly_2\text{-}His\text{-}Gly_2Tyr} - f[\theta]_{c\text{-}(Gly_2\text{-}Leu)_2} \quad (2)$$

Unlike *c*-Gly_5-Tyr, *c*-Gly_2-His-Gly_2-Tyr contains a contribution from the histidine in the 212-nm region. Hence it is difficult to determine the effect of the side chains on the amide n–π^* Cotton effect. As a result of the similarity of the 275-nm circular dichroism bands in *c*-Gly_5-Tyr and in *c*-Gly_2-Tyr-Gly_2-His, we will assume the perturbation of the tyrosyl side chain on the amide n–π^* transition to be the same in both compounds. Due to the similarity in their wavelengths, we have no simple way of estimating the coupling of the histidyl side chain to the amide n–π^* band. We will ignore this perturbation, assuming it to be small. Therefore, we use the same value of f(f = 2.8) in eq 2 as was used in eq 1. The difference circular dichroism curve (Figure 6) should give us the sum of the contributions of the two side chains.

This procedure leads to a 275-nm band in the difference curve which may be readily assigned to tyrosine. Also assigned to tyrosine is a broad peak, ([θ] = 1000) in the 230-nm region which is similar to that assigned tyrosine in *c*-Gly_5-Tyr. We also obtain a negative band ([θ] = 2600), at 211 nm which we assign as the contribution of the histidyl side chain which has an absorption band at that wavelength. The shorter

wavelength region is not easily amenable to interpretation by this procedure since it is composed of a mixture of the amide $\pi-\pi^*$ bands and the strong tyrosyl absorption in the 192-nm region.

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Presence of Arginine Residues at the Strong, Hydrophobic Anion Binding Sites of Bovine Serum Albumin*

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ABSTRACT: Derivatives of bovine serum albumin were prepared by partial chemical modification of the native protein with acetic anhydride, formaldehyde, and glyoxal. Rotational relaxation time, sedimentation velocity coefficient, and pH dependence of fluorescence polarization measurements indicate that the overall tertiary structure of the derivatives is the same as that of bovine serum albumin in the pH range from 4.5 to 10. However, the formaldehyde- and glyoxal-treated albumins with 30 and 80% modified arginine residues, respectively, have a binding affinity for 1-anilinonapthalene-8-sulfonate almost two orders of magnitude lower than the acetylated and native albumins. The results suggest that there are arginine residues at or close to the strong hydrophobic anion binding sites of bovine serum albumin.

For a long time serum albumin has been known to have a remarkable binding capacity for all kinds of cationic, anionic, and neutral ligands. The vast literature on this subject has been reviewed several times in the last three decades (Edsall, 1947; Klotz, 1953; Foster, 1960; Putnam, 1965).

Much of the work on the binding properties of serum albumin was carried out with ionic ligands having hydrophobic side chains. The affinity of the protein for such ligands was shown to depend on the hydrophobic character of the molecule as well as on the charged group. For example, increasing binding affinity was correlated with the length of the aliphatic chains of fatty acids and detergents. Also, serum albumin was shown to bind anions more tightly than cations or neutral molecules with similar hydrophobic groups, even at pH values where the protein itself was anionic. Numerous studies indicated that many ligands, in particular anionic ligands, had at least two classes of binding sites on serum albumin differing in binding affinity by one or two orders of magnitude. The strongest binding sites were, in most cases, less than ten and overlapped for a large number of similar ligands.

Beyond these general properties of the strong anion binding sites, very little is known about the structural characteristics of serum albumin which determine its unusual binding capacity. Except for the fact that the strong anion binding sites on serum albumin include cationic and hydrophobic amino acid residues, and indications that tryptophan residues are near the binding sites (Herskovits and Laskowski, 1962; Polet and Steinhardt, 1968; Swaney and Klotz, 1970), there is no direct information about the structure of these sites.

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