AROMATIC CONTRIBUTIONS TO CIRCULAR DICHROISM SPECTRA OF PROTEINS

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INTRODUCTION

Circular Dichroism (CD) is basically a type of spectrophotometry. In contrast to studies with unpolarized light, however, CD bands may be either positive or negative and are highly conformation dependent. Thus, the CD spectrum of a protein is potentially more diagnostic of its native conformation than is its ordinary absorption spectrum.

Initial CD studies of proteins dealt mainly with the region below 250 nm, in part, because these CD bands are much more intense than those in the region from 250 to 330 nm (near-UV). Recently, the availability of improved CD spectrophotometers has permitted extensive studies of near-UV CD bands in proteins. These bands may arise from tryptophanyl, tyrosyl, phenylalanyl, cystinyl, and certain prosthetic groups. Near-UV CD bands reflect tertiary structure, whereas far-UV CD bands usually indicate secondary structure.[1,2]

This review deals with the contributions of tryptophanyl, tyrosyl, and phenylalanyl side chains to the near-UV CD spectra of proteins. It covers literature published from 1967 to July, 1973. Earlier work has been reviewed by Beychok[3,4] and by Goodman and Toniolo.[5] Some current aspects of near-UV CD spectra have been reviewed by Sears and Beychok,[2] Adler et al.,[1] and Timasheff.[6]

Initial parts of my review present experimental and theoretical aspects pertinent to near-UV CD studies. Next, the characteristics of near-UV CD bands in model compounds are described and then used to analyze the spectra of selected proteins. The final section summarizes how near-UV CD spectra have been applied to problems in enzymology and protein chemistry.

EXPERIMENTAL ASPECTS OF NEAR-UV CD MEASUREMENTS

Presentation of Data

$\Delta\epsilon$ - Circular dichroism refers to the difference

in absorption of left and right circularly polarized light. Thus, the logical measure of CD intensity is in terms of molar absorptivity (molar extinction coefficient). CD intensity = $\Delta\epsilon \equiv \epsilon_L - \epsilon_R$, where ϵ_L is the molar absorptivity for left circularly polarized light, and ϵ_R is the value for right circularly polarized light. Modern CD spectrophotometers actually measure the difference in absorbance of left and right circularly polarized light, even though some are calibrated in other terms. The $\Delta\epsilon$ value can be calculated from the CD version of Beer's law.

$$\Delta A \equiv A_L - A_R = \Delta\epsilon cl,$$

where ΔA = absorbance for left circularly polarized light minus that for right circularly polarized light, c = molar concentration, and l = path length in cm.

Although physical and organic chemists have usually expressed CD intensities in terms of $\Delta\epsilon$ values, biochemists have generally used ellipticities. When plane polarized light passes through an optically active medium in the region of an absorption band, the light becomes elliptically polarized, owing to the unequal absorption of the left and right circularly polarized components.[3] Most CD instruments constructed in the first half of this century actually measured the amount of ellipticity produced by a solution.[7] Although modern CD instruments do not measure ellipticity, the earlier terminology has persisted in the biochemical literature. I believe the current use of ellipticity to describe CD is unfortunate, because this term obscures the basic physical process (unequal absorption) and adds unnecessarily complicated terminology and confusing units of measure (see also Reference 8, p. 74). Thus, I strongly recommend that CD data be expressed as $\Delta\epsilon$ values rather than as ellipticity values.

Should a solvent correction be applied to $\Delta\epsilon$? – Since the effective electric field strength of light is altered somewhat in a material medium, it would seem that the measured $\Delta\epsilon$ values should be corrected for the dielectric effect of the solvent or local environment upon the intensity. In practice, however, the major effects of solvent upon CD intensity are related to alterations in molecular conformation rather than changes of light intensity. Furthermore, there is little, if any, experimental support for the theoretical correction factor usually used for absorption processes.[9] Thus, application of a solvent correction factor for dielectric effects seems unwarranted at the present time.

Conversion of ellipticity to $\Delta\epsilon$ – Many of the data presented in this review were converted to $\Delta\epsilon$ values from the ellipticities given in the original publications.

$$\Delta\epsilon = [\theta]/3300$$

where $[\theta]$ is called the molecular or "molar" ellipticity, even though it has the units of degs cm^2 per *deci*mole.

Unfortunately, *near-UV* CD intensities of proteins have sometimes been expressed in terms of ellipticity per decimole of amino acid residue ($[\theta]_{MRW}$).

$$[\theta]_{MRW} = [\theta]/N$$

Averaging *near-UV* CD intensities over the total number of amino acid residues (N) is not justified, because only four types of amino acid side chains contribute CD in this region.

Rotatory strength – This quantity is often used to compare the intensities of CD bands having different shapes, e.g., sharp bands vs. broad bands. The experimental value of rotatory strength (R_s) may be obtained from the area under a CD band.

$$R_s = \frac{22.9 \times 10^{-40}}{\lambda_c} \int \Delta\epsilon \, d\lambda = 22.9 \times 10^{-40} \frac{C_1 C_2 A}{\lambda_c},$$

where A is area under CD band in cm^2, C_1 is conversion factor relating $\Delta\epsilon$ per cm of ordinate, C_2 is nm of wavelength per cm of abscissa, and λ_c is median wavelength of absorption band in nm. R_s will be in cgs units.

Operation of a CD Spectrophotometer

Figure 1 shows a simplified diagram of a CD spectrophotometer. The major differences from an ordinary spectrophotometer are the addition of a

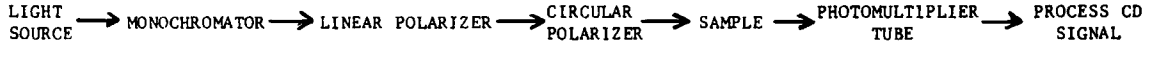

FIGURE 1. Simplified block diagram of CD spectrophotometer.

linear polarizer and a circular polarizer between the monochromator and the sample compartment. In terms of actual construction, many other changes are required to obtain reliable measurements of the small near-UV CD signals of some proteins ($\epsilon_L - \epsilon_R$ ranges from 10^{-3} to 10^{-5} of the average ϵ value). Technical aspects of designing a modern CD spectrophotometer have been described in detail by Velluz et al.,[8] Dratz,[10] Horwitz,[11] Hofrichter,[12] and Breeze and Ke.[13] Improvements described in the more recent papers should eventually become standard features on commercial instruments. In the following paragraphs, I give general comments which may help biochemists evaluate commercial instruments.

General considerations – First, there seems to be little justification for purchasing a combined ORD-CD instrument, because CD spectra can now be measured well into the vacuum-UV region.[14] Instruments designed specifically for CD tend to have better optical performance and are less expensive. Second, reflection or focusing of circularly polarized light should be avoided, because these processes may introduce spurious CD signals of appreciable size unless extremely precise optical elements are used. Third, major electronic advances have been made during the past few years and are likely to continue. Thus, processing of CD signals can be done more effectively and probably more cheaply than has been the case with most, possibly all, previous commercial CD spectrophotometers.

Light source – Most instruments have used 450-W xenon lamps. On the other hand, 75-W xenon lamps have given good results in several CD spectrophotometers. To my knowledge, no one has shown that the 450-W xenon lamps perform better than the 75-W lamps in a well-designed CD instrument. This point should be investigated more systematically, because the 75-W xenon lamps have several inherent advantages over the large lamps. The 75-W lamps are much cheaper, produce less heat, and have smaller power supplies.

Monochromator – For measurements in the near-UV and also far-UV region, a single prism monochromator is adequate even for the sharpest bands in proteins.

Circular polarizer – It alternately produces left and right circularly polarized light. The number of cycles per second from left to right and back to left circularly polarized light is called the "modulation frequency." Elimination of noise resulting from random movements of the xenon arc within the light source is best accomplished by using a high modulation frequency. Even with the most stable lamps, frequencies less than several thousand Hz are not high enough to completely eliminate noise from arc movements and other low frequency effects. High modulation frequencies, e.g., 50,000 Hz, minimize the noise and also permit using less stable lamps without sacrificing performance.

Circularly polarized light is produced either by electro-optic modulators (Pockels cell) or by piezo-optic (photoelastic) modulators. The advantages of photoelastic modulators* have been pointed out by Kemp[15] and by Breeze and Ke.[13] The photoelastic modulators operate at 25 to 50 kHz, have flat baselines, and give well-polarized light even when the incident beam is far from being parallel. In contrast, the electro-optical devices require exceptionally high quality crystals to obtain a flat baseline, are more difficult to operate at high frequencies, need an almost parallel light beam, and require very high operating voltages.

Sample compartment – Since current CD instruments use only a single beam, it is usually necessary to record separately the spectrum of the optically active sample and a reference sample. Pure solvent or buffer is generally used as the reference solution, although this practice can sometimes produce artifacts when measuring weak CD signals (see below).

Recently, commercial instruments have been developed which automatically subtract the reference spectrum even though only a single beam is used (see Reference 16 for a summary). The method for obtaining these difference CD spectra involves inverting the direction of circular polarization after the beam passes through the sample cuvet, and then passing it through the reference cuvet before it strikes the photomultiplier tube. Chau and Yang[16] found, however, that several commercial instruments gave false CD values when using the difference CD mode for samples having strong absorption and weak CD bands. Unfortunately, these are just the conditions under which

*Performance of older commercial instruments can be improved significantly by installing a photoelastic modulator and the electronics necessary to process the high frequency CD signal.

difference CD measurements are most needed to minimize the noise. Apparently, difference CD spectra still need to be obtained by subtracting CD spectra recorded separately, as has been done for the difference CD spectra described later in this review.

Processing CD signal – Extracting the small CD signal from the far larger background noise is best accomplished with a phase sensitive detector which locks onto the CD signal.[10] The resulting signal may then be fed to a signal averager to obtain more reliable low-noise CD spectra. For those doing extensive near-UV CD measurements on proteins, signal averaging is probably a definite asset.

There are two types of signal averaging which may be classified according to their input signal: either digital or analog. Analog averaging of CD spectra was introduced by Dratz[10] and later used by Myer and McDonald[17] and Horwitz et al.[18] Usually, the spectrum is displayed continuously as the data accumulate so that averaging may continue until the desired signal-to-noise ratio is obtained. Wavelength scan controllers permit automatically averaging any number of spectra. In addition, a signal averager may be used to subtract the reference baseline from the sample CD trace.[17] However, I do not recommend this subtraction in most uses. When a low signal-to-noise ratio exists, this subtraction will increase the noise in the final record and will tend to obscure weak CD fine structure. For high signal-to-noise ratios, the baseline is likely to be flat and thus may not need to be subtracted anyway.

Hooker and Schellman[19] presented an example of digital signal averaging. In this case, the digitized CD data are transmitted on line to a digital computer which has been programmed to process the information in the desired way. When only a few types of experiments are being repeated many times, signal processing by the digital computer may be a great convenience, because only a few programs are required. For example, the CD spectra may be plotted as $\Delta\epsilon$ values vs. wavelength. In the ultimate case, the digital computer might present the CD spectrum and also a breakdown into individual contributions from different amino acid side chains. On the other hand, if many different types of measurements and data analysis are required, analog signal averaging may be more convenient.

CD Artifacts

When measuring small CD signals, the possibilities for artifacts are great. Thus, each CD spectrophotometer should be periodically tested for artifacts. The most important test will detect absorption-dependent artifacts. Measure the apparent CD of a nonoptically active sample* having the same absorbance as the optically active sample. If the nonoptically active compound has a trace different from that of the solvent, an artifact exists. Absorption-dependent artifacts may result from reflections, from stray light, and from inadequate control by the servo system regulating the photomultiplier voltage. Probably, some published CD spectra have been vitiated by absorption-dependent artifacts, because few investigators have reported testing for this artifact. As mentioned previously, absorption-dependent artifacts have been reported in differential CD measurements on several commercial instruments.[16] Two other possible examples of this artifact will be given in this section on Aromatic CD Bands in Model Compounds.

Other artifacts can result when attempting to measure the CD spectrum of oriented samples. Orientation artifacts may occur with films,[20,21] with crystals,[22] with photosensitive compounds in frozen solutions,[23] and with other anisotropic samples.

Low Temperature CD Measurements

Recording spectra at low temperatures is often an excellent way to sharpen fine structure CD bands and to detect conformational motility. Thus, cooling sometimes leads to major changes in the CD spectrum, especially for nonrigid aromatic compounds. Unfortunately, such CD changes may also result from altered intermolecular interactions (dimers or other aggregates) and possibly from orientation effects. If cooling does appreciably alter the CD spectrum, supplementary studies may permit identifying the mechanism(s) involved.

Two methods are available for obtaining low-temperature CD spectra: measurements on solutions above their freezing points and measurements on frozen solutions (glasses). The success of both methods depends upon finding a suitable

*DL-amino acids, *p*-cresol, 3-methylindole, toluene and related compounds are useful for this test.

solvent, e.g., water-glycerol (1:1/v:v) buffers for proteins[24] and certain organic solvents for low-molecular weight amino acid derivatives.[25] Each method has certain advantages the other lacks. Measurements on glasses can be done at a lower temperature (down to 77°K) but may be more likely to suffer from extraneous effects, such as aggregation (dimers, trimers, etc.). With water-glycerol glasses, the solute concentration must be unusually large, because the path length must be kept at 0.2 mm or less to obtain satisfactory glasses, i.e., no depolarization and small baseline offset.[24,25] In contrast, solution CD measurements may be made at much lower concentrations, thereby minimizing the possibility of aggregation upon cooling.

Application to proteins – The first step is to find out if the protein under study has a similar conformation in both the water-glycerol buffer and the fully aqueous buffer. This may be done by comparing CD spectra in the two solvents at room temperature. If adding glycerol does not alter the CD spectrum by more than a small amount, the native conformation of the protein is probably not altered much by the glycerol. Small changes in the near-UV CD intensity do not necessarily imply an altered protein conformation, because glycerol slightly increases the absorption intensity of "exposed" aromatic side chains and causes a small red shift.[26,27]

Variable temperature CD measurements on protein solutions down to about 240°K may permit detecting conformational motility, if any is present.[28] If changes in rotatory strength occur upon cooling, these measurements should be made at both high and low concentrations to verify that the observed CD changes are not concentration-dependent. If the changes in rotatory strength are not dependent upon concentration, then these changes are not likely to result from aggregation. In the absence of aggregation, motility may be studied at even lower temperatures by using a short path length.

CD spectra of proteins at 77°K provide the best information about the positions of individual CD bands. If aggregation and other artifacts are absent, the 77°K CD spectrum should resemble the CD spectrum observed at room temperature except for having sharper and somewhat more intense CD bands. Radical changes in rotatory strength and/or positions of the CD bands upon cooling a protein to 77°K suggest that some type of artifact may have occurred.

CD measurements may be made easily at 77°K, if one has a suitable spectroscopic dewar and a CD instrument modulated at 1 kHz or more.[11] The protein solution is placed in a suitable cuvet and then inserted into the spectroscopic dewar filled with liquid nitrogen. The rate of freezing can be controlled by the speed of lowering the cuvet holder into the liquid nitrogen. For some proteins and buffer solutions, the fastest possible freezing gives better glasses, whereas the converse has been observed for other proteins. Finding the best conditions for a specific protein is mainly done by trial and error.

THEORETICAL BASIS FOR AROMATIC CD BANDS

Absorption Bands

Since CD is an absorption process, knowing the absorption spectrum of a chromophore facilitates understanding its CD spectrum. The aromatic bands described in this review involve the transition of an electron from a filled π orbital (ground state) to an empty π^* orbital having a higher energy (excited state). Each aromatic ring has several of these $\pi-\pi^*$ electronic transitions, some of which occur in the near-UV and others in the far-UV. The different $\pi-\pi^*$ transitions are designated by a notation based upon certain properties of their excited states, e.g., ^1L_b, ^1L_a, ^1B_b, and ^1B_a.[29]

Vibronic transitions – Electronic movements during excitation are usually also accompanied by changes in the vibrational energy of the nuclei. The combination of vibrational and electronic excitation by light has been termed a "vibronic transition." For each electronic excited state of an aromatic amino acid side chain, there are several vibronic transitions; i.e., an electron from the ground electronic level may be excited to any one of several excited states differing only in their vibrational energies (see Figure 2, left). In addition, at room temperature a small portion of the molecules in the ground electronic state will be vibrationally excited by heat in accordance with the Boltzmann distribution law. Transitions can also occur from these states to the various excited states (see Figure 2, right).

Each vibronic transition is designated by

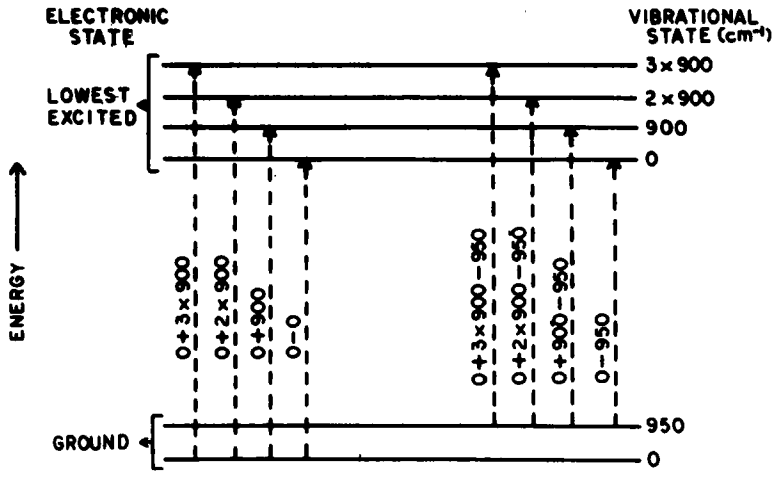

FIGURE 2. Illustration of a vibronic transition.

numbers showing which vibrational states are involved in the excitation. For example, 0–0 refers to a transition from the 0 vibrational state in the ground electronic level to the 0 vibrational state in the excited electronic level; 0 + 900 cm^{-1} indicates a transition from the 0 vibrational state in the ground electronic level to the 900 cm^{-1} vibrational state in the excited electronic level. More details on vibronic transitions are given by Suzuki.[30]

Solvent effects – From the description given above, one would expect an absorption spectrum to consist of lines corresponding to the energies of the several vibronic transitions, as is actually observed for some aromatic compounds in the vapor phase. When these same moieties are dissolved in solvents or located within a protein, they interact in varying degrees with the neighboring molecules or residues, causing the spectrum to be blurred.[31] These interactions are greatest with polar molecules, e.g., water, and least with nonpolar ones, e.g., saturated hydrocarbon side chains or solvents. In addition to electrostatic interactions, hydrogen bonding may occur between the solvent and the solute. Owing to the difference between the solute-solvent interaction energies for the ground and the excited electronic states, the wavelength positions of the vibronic transitions are shifted somewhat relative to the positions observed in the vapor state. If the solvent decreases the energy of the excited state more than that of the ground state, the absorption bands shift to the red. In the converse situation, the bands will be shifted toward shorter wavelengths.[9]

When aromatic amino acids are dissolved in water or other polar solvents, a multitude of different solvent-solute interactions occur. Many solute molecules may have their 0–0 vibronic transitions at slightly different wavelengths from the average position. Thus, the observed absorption spectrum is broadened, which may obscure the individual vibronic transitions.

Temperature effects – Cooling sharpens the absorption bands of many aromatic molecules. Partly, the sharpening is due to the reduction of molecular motion, which decreases the number of different types of solvent-solute interactions. Cooling also depopulates the vibrationally excited states of the ground electronic level, thereby reducing the intensity of their transitions (see Figure 2). The latter effect can cause much sharpening, if the vibrations have low energies.

Dipole strength and electronic transition moment – The dipole strength (D_s) is proportional to the total intensity of an electronic absorption band and can be obtained from the area under that band.[32]

$$D_s = \frac{9.18 \times 10^{-39}}{\lambda_c} \int \epsilon d\lambda,$$

where ϵ is molar extinction coefficient, λ_c is median wavelength of band, and D_s is in cgs units.

The electronic transition moment (μ), which is needed for calculating CD intensities, gives the size and direction of a transition.

$$|\mu| = \sqrt{D_s}$$

TABLE 1

Mechanisms for Inducing Optical Activity in an Electronic Absorption Band of Group 1 by its Interaction with Group 2[a]

Mechanism[b]	Group 1 contributes[c]	Group 2 contributes[c]	Distance dependence[d]
One Electron	μ_A, m_B	Static electric field[e]	Varies with symmetry of chromophore and type of perturbing field[f]
μ–m (electric-magnetic coupling)	μ_A	m_β	R^{-4}
μ–μ (electric dipole-electric dipole coupling; dipole-dipole coupling; Kirkwood-Kuhn coupled oscillator)	μ_A	μ_β	R^{-2}

[a]Modified from Schellman.[33]
[b]Alternate terminology given in parentheses.
[c]μ, electric dipole transition moment for absorption bands designated by subscript; m, magnetic dipole transition moment.
[d]R, distance between centers of two groups.
[e]Perturbing group not effective if lying in a symmetry plane of the chromophore.
[f]For indolyl ring (C_s symmetry), R^{-2} for point charge and R^{-3} for dipole; for phenyl and phenolic ring (C_{2v} symmetry), R^{-3} for point charge and R^{-4} for dipole.[36]

The direction of μ can be measured by using oriented crystals. The electronic transition moment has the same units as an electric dipole, i.e., charge times length. Thus, μ is often represented by a single vector located at the center of the chromophore (dipole approximation). For calculating CD intensities, however, μ should be represented by distributing positive and negative charges over the entire chromophore, the so-called monopole approximation.

CD Theory

Since the mechanisms giving rise to CD bands have been lucidly summarized[33] and also described in detail,[2,34-38] this review describes only those theoretical aspects that are especially relevant to near-UV CD. If a chromophore does not have a plane of symmetry or a center of inversion, it may be inherently optically active, e.g., the disulfide chromophore of cystinyl. For chromophores having a plane of symmetry or a center of inversion, CD may occur only if these chromophores are perturbed by their surroundings in an appropriate way. Thus, the aromatic rings of tyrosyl, tryptophanyl, and phenylalanyl gain their near-UV CD bands through interactions with the amino acid moiety or with nearby groups in a protein.

Table 1 summarizes the mechanisms by which a dissymmetric environment may induce CD bands. Although the strongest CD bands tend to occur when the interacting groups are juxtaposed, a chromophore can still be influenced by groups located some distance away. The μ–μ coupling mechanism may possibly even be effective for separations as great as 10 to 15 Å, if the electronic transitions are intense.[39] On the other hand, the one electron mechanism and μ–m coupling tend to be shorter range effects, possibly requiring that the two groups be almost in van der Waals' contact. For electronic transitions having molar extinction coefficients greater than 1,000, μ–μ coupling may be expected to contribute significantly to the observed CD bands.[33]

μ–μ coupling – Since this interaction seems to be the major mechanism contributing to near-UV tyrosyl and tryptophanyl CD bands (see below), we shall consider it in more detail. The μ–μ mechanism involves an interaction (coupling) between two electronic transitions in different groups; e.g., the near-UV tyrosyl transition of one side chain may couple with a far-UV transition of

a nearby tyrosyl or phenylalanyl side chain. The absorption properties of a particular chromophore are influenced to varying extents by the other chromophores belonging to that molecule, especially in the case of proteins.

A somewhat simplified physical picture may facilitate understanding these interactions. Suppose that a tyrosyl side chain of protein initially absorbs a near-UV photon. Within a time interval of about 10^{-15} s, the excitation energy may be transferred to another chromophore and then back to the tyrosyl side chain, in accordance with the uncertainty principle ($\Delta E\ \Delta t \geqslant \hbar$). Since the other chromophore is excited for only a short time, its excitation energy (absorption wavelength) may differ appreciably from that of the photon initially absorbed by the tyrosyl side chain. The difference in their absorption wavelengths serves only to decrease the extent of transfer. This transfer of excitation energy causes electrical interactions between the chromophore being excited and the one giving up this energy, because the distribution of electrons within a chromophore is altered by excitation and de-excitation. If the geometric arrangement of the two electric transition moments is favorable, CD may be generated, in accord with the approximate equations given in Reference 33. These same interactions also cause small changes in the absorption intensities, i.e., the dipole strengths.[37,40]

When μ–μ coupling occurs, each of the two transitions gains rotatory strength as a result of the interaction. These induced rotatory strengths have the same absolute size but differ in sign. Thus the μ–μ interaction produces two opposite signed CD bands, one at each of the two absorption bands. The longer wavelength band may be either positive or negative and has varying CD intensity, determined by the actual geometry of the interacting transition moments. The apparent spectral characteristics of μ–μ coupling are influenced by the energy separation between the two absorption bands. Usually, μ–μ coupling is subdivided into the degenerate and nondegenerate cases, since different equations are used for these theoretical calculations.[33]

Nondegenerate case of μ–μ coupling – To a first approximation, these induced CD bands have the same shape as their absorption bands. The induced CD intensity is approximately proportional to the product of the dipole strengths of the two coupled transitions but is diminished when the energy separation between the two bands is large. Judging from the intensities and wavelengths of electronic transitions in proteins,[27,39,41,42] the near-UV transitions of aromatic side chains may tend to couple most strongly with the transitions of other aromatic side chains and the peptide π–π^* transition at 190 nm. When a protein contains prosthetic groups or other bound molecules, coupling with their transitions is also possible.

Exciton CD bands – In the so-called degenerate case where both transitions would have the same energy if the chromophores did not interact, μ–μ coupling usually is referred to as an "exciton effect." The interaction, which is approximately proportional to the dipole strength, shifts one band to higher energy and the other to lower energy. If the interaction (splitting) between the two transitions is small, their opposite signed CD bands will occur at nearly the same wavelength, causing almost complete cancellation. When the interaction is larger, the two CD bands spread apart more, which reduces their cancellation. The resulting CD spectrum is obtained by adding the individual positive and negative CD bands. The original pair of CD bands has about the same shape as the absorption band of the monomer, and their intensities are proportional to the dipole strength. Tinoco[43] has given more details on exciton CD bands.

Calculating near-UV CD intensities of aromatic side chains resulting from μ–μ coupling – When the atomic coordinates of a protein are known for the crystalline state, an approximate calculation of near-UV CD may be feasible.[41,42] The calculation of rotatory strength also requires accurate values for the interaction energies between transitions and their directions within the chromophore. The most reliable way to obtain the transition moment directions is usually by experiment or by symmetry considerations, where possible. Interaction energies should be calculated using the distributed monopole approximation, because the dipole approximation fails for the close separations that are most important in proteins.[41] Woody and his co-workers[41,44,45] have calculated the transition monopoles for many chromophores found in proteins. The reliability of these monopoles is crucial for accurate CD calculations. If the transition monopoles are unreliable, the calculated

rotatory strength may not even have the correct sign.

Effects of cooling – The observed CD spectrum is the population-weighted average of all conformers present in solution. Especially with some low-molecular weight amino acid derivatives, many conformers may be in equilibria. Some conformers may have positive CD bands, while others may have negative bands. Thus, extensive cancellation between positive and negative CD bands is possible. Often, the CD intensity (rotatory strength) may be greatly enhanced by cooling. At low temperatures the lower energy conformations become more heavily populated in accordance with the Boltzmann distribution law,[46,47] thereby tending to reduce cancellation between conformers with opposite CD signs. Cooling may also alter solvent-solute interactions, which may affect the CD spectrum of each conformer and may alter the distribution of conformers. Even in the absence of an altered rotatory strength, the CD bands at low temperatures will be sharpened, giving an increased $\Delta\epsilon$, if the absorption bands become sharpened.

Distortion of CD spectrum by multiple species – As a matter of convenience, I shall refer to each conformer or conformer-solvent unit having a unique CD spectrum as a *species*. When multiple species exist, they may distort the shape of the CD spectrum from that observed for a single species.[48] In addition, the occurrence of multiple species may produce a mismatch between the wavelength positions of the CD and absorption bands.[49] Usually, each species has about the same absorption intensity, but may have its wavelength position shifted. On the other hand, the CD intensity is highly variable. Thus, each species may contribute a much different proportion of the observed CD and absorption spectra. If the species with positive CD mostly have their bands at either longer or shorter wavelengths than those with negative CD, the experimentally observed CD spectrum may even show both a positive and a negative region within a single absorption band. This kind of CD spectrum occurs most frequently when the wavelength position of the absorption bands is easily shifted by solvent-solute interactions, as is the case with the ^1L_b tyrosyl and the ^1L_a tryptophanyl bands. Similar effects may occur in proteins having several tyrosyl or tryptophanyl residues, e.g., two tyrosyl side chains having their absorption bands at different wavelengths and having opposite CD signs.

AROMATIC CD BANDS IN MODEL COMPOUNDS

Since the CD sign and intensity are determined by the side chain orientation and interactions with its surroundings, an extensive catalogue of CD spectra is required to determine the range of intensities to be expected in proteins. Owing to their commercial availability, *N*-acetyl amides and esters of aromatic amino acids tend to be the most studied models for the aromatic side chain CD bands of proteins. At room temperature, however, these models are usually much less rigid than are the side chains in native proteins. Thus, the *N*-acetyl amides and esters usually have weak CD intensities comparable to those observed for denatured proteins with motile side chains (see *Effects of denaturing agents*). Cyclic dipeptides (diketopiperazines) are generally much more rigid even at room temperature and may have aromatic CD intensities comparable to those observed for native proteins.[50-52]

The wavelength position and sharpness of aromatic CD bands in proteins is often strongly influenced by the environment of each side chain e.g., hydrogen bonding, polar or charged groups, and polarizability.[9] Although some groups on the surface of a protein may be exposed mainly to water, most side chains have extensive contacts with other moieties. Buried aromatic side chains may exist in a variety of environments ranging from nonpolar (e.g., surrounded by valyl or leucyl side chains) to polar (e.g., surrounded by carboxy or hydroxy groups). In many cases, model compounds dissolved in organic solvents closely reproduce the wavelength position and sharpness observed for the CD bands of side chains within native proteins.

Near-UV Phenylalanyl CD Bands

Vibronic transitions – This phenylalanyl absorption band (^1L_b) is closely related to the very weak near-UV band observed for benzene.[25,27,53] The high symmetry of benzene causes the weakness, a so-called symmetry forbidden band. The bands occurring in benzene arise because "nontotally symmetrical" nuclear vibrations perturb the symmetry of the electronic

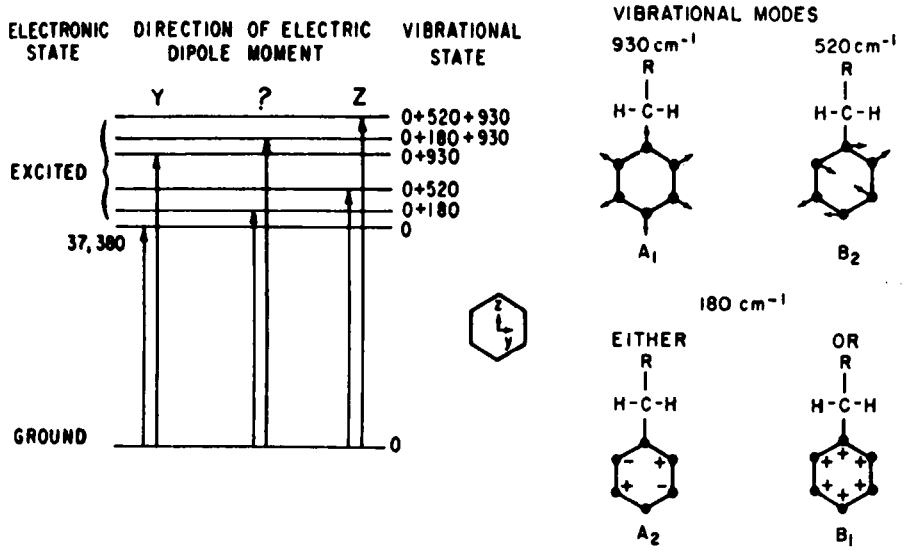

FIGURE 3. Major transitions occurring in the near-UV CD spectra of phenylalanyl side chains. The y and z axes lie in the plane of the phenyl ring, as indicated in diagram. The x axis is perpendicular to the ring. Plus and minus signs for A_2 and B_1 vibrations refer to motion along x axis; arrows are in the yz plane. $\mu_z = \mu_y = 0.27 \times 10^{-18}$ cgs.[44] (Reprinted from Horwitz et al.,[25] *J. Am. Chem. Soc.*, 91, 188, 1969, © 1969 by the American Chemical Society. Reprinted by permission of the copyright owner.)

states. In the case of phenylalanyl, the amino acid substituent perturbs the symmetry of the phenyl chromophore only weakly. Thus, the ^1L_b phenylalanyl absorption band results both from the effects of the substituent and from the influence of nontotally symmetrical vibrations.

These nontotally symmetrical vibrations may drastically alter the CD intensities of their vibronic bands.[25] In fact, the transitions involving a single, nontotally symmetrical vibration may even have a different CD sign from the other transitions. Theoretical aspects of vibronic coupling have been discussed.[54-56]

Figure 3 summarizes the 3 series (progressions) of vibronic transitions that have been identified in phenylalanyl CD spectra.[25] Each progression is built upon a starting vibronic transition whose properties are affected by any involvement of a nontotally symmetrical vibration, e.g., 0–0 (no vibrations), $0 + 180$ cm^{-1} (A_2 or B_1 nontotally symmetrical vibration), $0 + 520$ cm^{-1} (B_2 nontotally symmetrical vibration). The remaining bands of each progression arise from adding the energy needed to excite the totally symmetrical ring breathing vibration (930 cm^{-1}). The first two members of each progression are thus about 6 nm apart and have comparable CD intensities of the same sign. Each progression, however, may have a different CD sign. Similar vibronic transitions have been observed in the CD spectra of other compounds containing the phenyl chromophore.[57,58]

Typical CD spectra in model compounds – The phenylalanyl CD spectra may have a variety of appearances, depending upon the signs and relative intensities of each progression. The spectra shown in Figures 4 to 6 are typical of phenylalanyl compounds[25,59] (names abbreviated in accord with recommendations of IUPAC-IUB commission).[60] These CD bands occur between about 268 and 250 nm, with the longest wavelength band being between 264 and 268 nm. The latter band rises sharply from the baseline, even at room temperature. There are at least two prominent vibronic bands of the same sign separated by about 6 nm. If alternating positive and negative CD progressions occur, the phenylalanyl bands appear especially sharp (see Figure 4). The wavelength position of the phenylalanyl 0–0 band is shifted slightly by changes in the solvent properties.[25,61]

Range of CD intensities for phenylalanyl model compounds – The rotatory strengths of phenylalanyl CD bands are highly conformation-dependent. For example, cooling Ac-Phe-NH_2 to

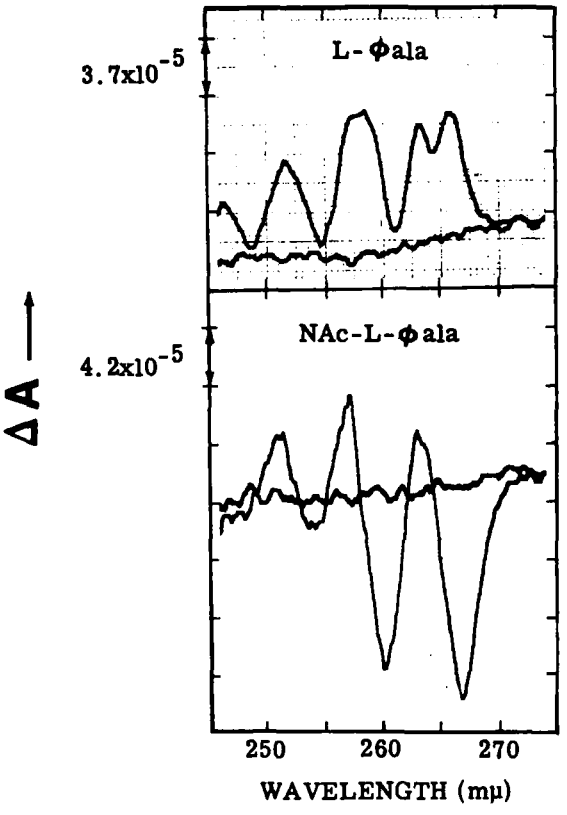

FIGURE 4. CD records of 5.8 m*M* L-phenylalanine (top) and of 4.6 m*M* Ac-Phe (bottom) in water (pH 6) at 298°K. Path length, 1.0 cm. (Reprinted from Horwitz et al.,[25] *J. Am. Chem. Soc.*, 91, 186, 1969, © 1969 by the American Chemical Society. Reprinted by permission of the copyright owner.)

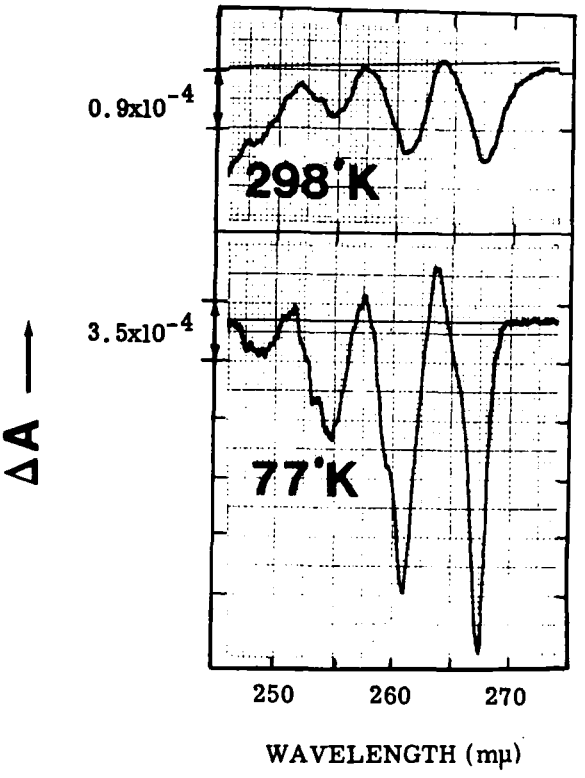

FIGURE 5. CD traces of Ac-Phe-NH_2 dissolved in methanol-glycerol (9:1, v:v) at 298°K (220 m*M*) and at 77°K (275 m*M*). Path length, 0.2 mm. Base lines are indicated by straight lines. (Reprinted from Horwitz et al.,[25] *J. Am. Chem. Soc.*, 91, 186, 1969, © 1969 by the American Chemical Society. Reprinted by permission of the copyright owner.)

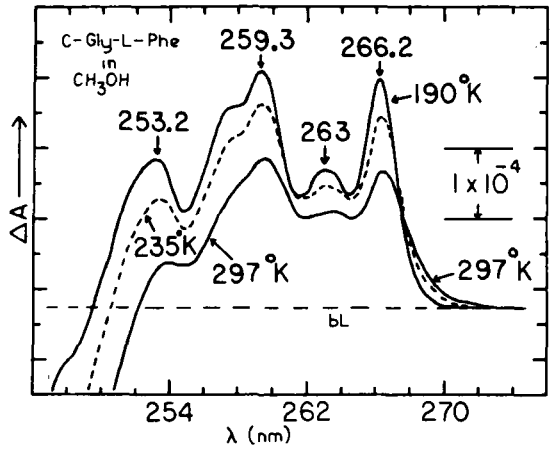

FIGURE 6. CD spectra of *cyclo*(-Gly-Phe-) dissolved in methanol at 297°K (4.60 m*M*), 235°K (4.95 m*M*), and 190°K (5.22 m*M*). Path length, 0.5 cm. bL indicates base line. (From Strickland, Wilchek, and Billups.[59])

77°K (Figure 5) causes a 6.6-fold increase of rotatory strength (data in Reference 25 corrected for solvent contraction[62]). Furthermore, Pino et al.[63] have shown that a helical copolymer containing a phenyl side chain has about 16 times the rotatory strength observed for the monomeric phenyl derivative.

To assess the possible sizes of the phenylalanyl CD bands of proteins, the $\Delta\epsilon$ values are useful. Table 2 summarizes the maximal $\Delta\epsilon$ values observed for numerous phenylalanyl derivatives.[64-71] From these values, one can expect the $\Delta\epsilon^{max}$ to range from about +0.3 to -0.3 M^{-1}_{Phe} cm^{-1} per phenylalanyl side chain at room temperature (equivalent to $|\Delta\epsilon^{max}|f\epsilon^{max} \leqslant 1.4 \times 10^{-3}$). With nonrigid phenylalanyl derivatives, e.g., Ac-Phe-NH_2 and linear dipeptides at room temperature, the absolute value of $\Delta\epsilon^{max}$ does not exceed 0.06 M^{-1}_{Phe} cm^{-1}. In contrast, one cyclic hexapeptide has a $\Delta\epsilon^{max}$ of 0.28 M^{-1} cm^{-1} per

TABLE 2

Near-UV CD Intensities of Selected Phenylalanyl Model Compounds at Room Temperature, unless Otherwise Indicated

Compound[a]	Solvent[b]	$\Delta\epsilon_{Phe}^{max}$[c]	λ^{max}, nm	Reference
Phe	H_2O (pH 7)	0.018	258	25, 64, 65
Ac-Phe-NH_2	H_2O (pH 7)	−0.050	267	61, 66, 67
	8 *M* guanidine-HCl	−0.06	268	67
	M-G	−0.04	268	25
Ac-Phe-NH_2 (rigid)[d]	M-G	−0.24	268	d
Ac-Phe-NH_2 (monomer)	EPA (77°K)	−0.37[e]	267	25
Ac-Phe-NH_2 (aggregate)	EPA (77°K)	0.3[e]	268	25
N-stearyl-Phe *n*-hexyl ester	CH_3OH	−0.04	267	59
	isopentane	0.03	258	59
Phe-Gly	KPi	0.016	259	65
Gly-Phe	KPi	−0.036	267	65, 68
cyclo(-Gly-Phe-)	CH_3OH	0.09	260	59
cyclo(Gly-Leu-D-Phe--Gly-Leu-D-Phe-)	CF_3CH_2OH	0.17	267	69
	CF_3COCF_3	0.28	261	69
cyclo(-Gly-D-Phe-Leu--Gly-D-Phe-Leu-)	CF_3COCF_3	0.03	261	69
cyclo(-Val-Pro-Pro-Ala--Phe-Phe-Pro-Pro-Phe--Phe-)	CH_3OH-H_2O (1:1)	−0.13	261	70
poly-Phe	$CHCl_3$-CF_3COOH	−0.05	262	71
Copolymer containing Ph[f]	$CHCl_3$	−0.27	262	63
Monomer unit[g]	$CHCl_3$	0.016	262	63

[a] Abbreviations defined in Reference 60.
[b] Abbreviations: M-G, methanol-glycerol (9:1, v:v); EPA, ethyl ether-isopentane-ethanol (5:5:2, v:v:v).
[c] $\Delta\epsilon_{Phe}^{max}$, maximal $\Delta\epsilon$ value per phenylalanyl in units of M_{Phe}^{-1} cm^{-1}.
[d] $\Delta\epsilon$ for rigid Ac-Phe-NH_2 at room temperature was estimated by multiplying $\Delta\epsilon$ observed at room temperature times the increase in rotatory strength upon cooling to 77°K.
[e] Data in Reference 25 corrected for solvent contraction upon cooling.
[f] Copolymer of (*R*)-3,7-dimethyl-1-octene with styrene.
[g] (3*S*, 9*S*)-3,9-dimethyl-6-phenylundecane was used as model of monomer unit.

phenylalanyl. Blaha et al.[69] suggested that this intense CD results because the two phenyl rings of *cyclo*(-Gly-Leu-D-Phe-Gly-Leu-D-Phe-) are stacked.

Mechanisms generating phenylalanyl CD – Recently, Woody[44] calculated the CD intensities arising from μ–μ coupling for several rigid conformations of poly-Phe. His largest calculated ^1L_b CD intensity appreciably exceeds the largest experimental value observed for phenylalanyl derivatives (see Table 2). Apparently phenylalanyl ^1L_b CD intensities may be at least partly determined by μ–μ coupling with other groups having strong electronic transitions. Exciton interactions between nearby phenylalanyl side chains are not likely to be important, since their ^1L_b transitions are so weak. Some influence from static perturbations is possible, but the C_{2v} symmetry of the phenyl ring reduces their effectiveness.

Near-UV Tyrosyl CD Bands

Vibronic transitions – The hydroxy substituent effectively reduces the symmetry of the phenyl chromophore so that the ^1L_b band of tyrosyl is about eight times more intense than the corresponding band of phenylalanyl. This large enhancement overshadows the small intensity contributed by the nontotally symmetrical vibrations,[72] which are so significant in phenylalanyl spectra. In fact, the effects of nontotally symmetrical vibrations are not readily detected in the CD or absorption spectra of tyrosyl.[49] The major vibronic transitions of the tyrosyl side chain

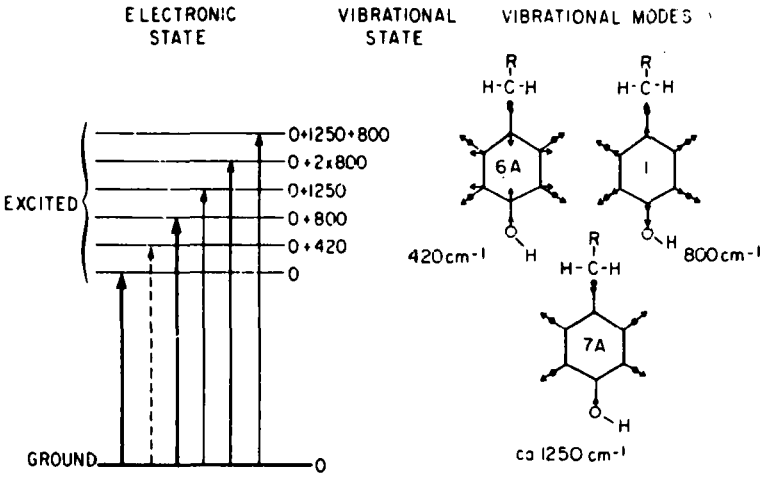

FIGURE 7. Major transitions occurring in the CD and absorption spectra of tyrosyl side chains. The 0-0 and 0 + 800 cm^{-1} transitions are most intense. The 0 + 420 cm^{-1} transition is very weak. $\mu \approx 1.2 \times 10^{-18}$ cgs. (Reprinted from Horwitz et al.,[49] *J. Am. Chem. Soc.*, 92, 2125, 1970, © 1970 by the American Chemical Society. Reprinted by permission of the copyright owner.)

involve totally symmetric vibrations (see Figure 7). Both symmetry considerations[25,49] and experimental evidence[73] indicate that these ^1L_b vibronic transitions are polarized in the plane of the phenolic ring and perpendicular to the line joining the oxygen and the methylene substituents. These bands are best resolved in organic solvents, especially at low temperatures.[49,74] Even at room temperature using an aqueous solvent, however, the 0–0 transition may be observed as a shoulder occurring about 6 nm to the red side of the absorption or CD maximum (0 + 800 cm^{-1}). Similar vibronic bands have been observed in spectra of *O'*-methyl-tyrosyl compounds.[74]

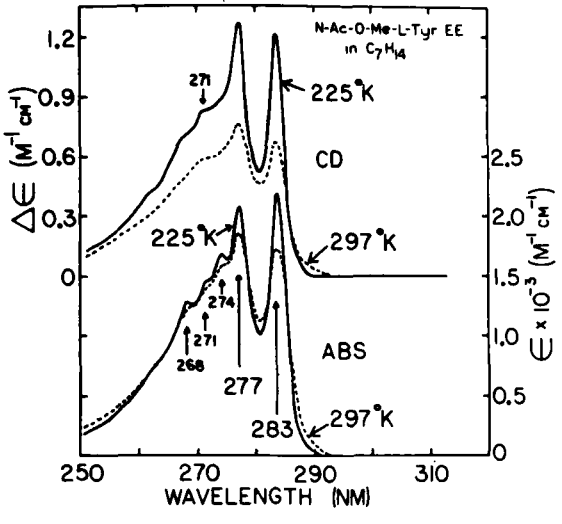

FIGURE 8. CD and absorption spectra of Ac-Tyr(Me)-OEt dissolved in methylcyclohexane at 297 and 225°K. (From Strickland et al.,[74] *J. Biol. Chem.*, 247, 574, 1972. With permission.)

Altering the solvent composition does not affect the spacing between vibronic bands but may shift the position of the 0–0 band by as much as 6 or 7 nm. Hydrogen bonding,[74] polarizability,[27,75] and polar groups[9] are probably the main solvent properties that determine the band position. Bonding the phenolic hydroxy group to a hydrogen acceptor group may cause red shifts as large as 4 nm. The greatest red shifts have been observed for hydrogen bonding to the peptide carbonyl oxygen[74] and the imidazole >N,[76] although even larger red shifts may occur for binding to the carboxylate acceptor.[77,78] Nearby polar groups in a protein may cause either long or short wavelength shifts, depending upon their orientation, because the ^1L_b excited state of tyrosyl is more polar than the ground state.[79,80]

CD spectra of compounds containing only one tyrosyl – For tyrosyl compounds existing mainly as a single conformer, the ^1L_b CD band has almost exactly the same shape as its absorption band (see Figure 8).[52,74] The CD spectrum, of course, may be either positive or negative, depending on the conformation.

Many tyrosyl model compounds exist as several

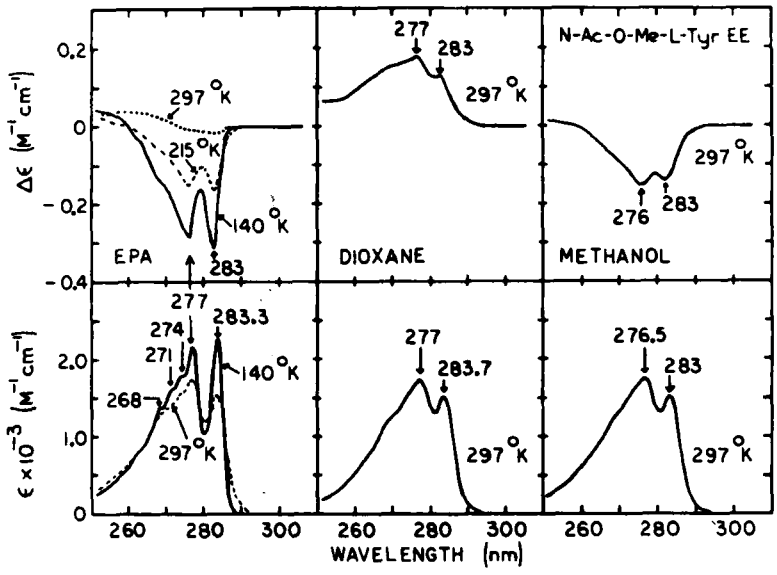

FIGURE 9. CD and absorption spectra of Ac-Tyr(Me)-OEt dissolved in EPA, dioxane, and methanol. (From Strickland et al.,[74] *J. Biol. Chem.*, 247, 574, 1972. With permission.)

conformers or species that are in equilibria. Each species may be expected to have a CD spectrum with shape similar to that of its absorption spectrum. Apparently, multiple species cause the distortion observed in the CD spectra of Ac-Tyr-OEt[49] and Ac-Tyr(Me)OEt dissolved in EPA at 297°K (see Figure 9). These species must be different conformers, since the CD spectra of L-tyrosine ethyl ester and tyrosyl diketopiperazines in EPA are not distorted.[52] At least two conformers seem to exist in EPA at 297°K: one with its absorption spectrum slightly red shifted and having negative CD bands, the other having positive CD bands. For example, Ac-Tyr-OEt and Ac-Tyr(ME)OEt give positive CD bands in dioxane and negative bands in methanol (see Figure 9). These spectra may simulate two populations of tyrosyl conformers in EPA.[49] A reversal of sign may be seen also within the ^1L_b CD band of *cyclo*(-Gly-His-Gly-Tyr-Ala-Gly-) dissolved in water (Figure 2 in Reference 81).

These examples illustrate that the CD maximum may be shifted considerably from the wavelength of the absorption maximum when the various species have opposite CD signs. In these extreme cases, the CD intensity is relatively small due to the large cancellation in the region of overlapping bands.

CD spectra of compounds containing two or more tyrosyls – The appearance of these spectra depends upon whether all tyrosyl sites are rigid and identical. If not, these CD spectra may resemble those described for multiple species of compounds having only a single tyrosyl. When both species with a positive CD and species with a negative CD are present, the shape of the resulting CD spectrum may resemble that given by an exciton interaction.

Strickland et al.[52] presented evidence that *cyclo*(-Tyr-Tyr-) in EPA at 140°K exists mainly as a single conformer having two identical side chain orientations. An approximate resolution of this CD spectrum reveals the exciton CD contribution superimposed upon the "normal" tyrosyl CD resulting from other interactions (see Figure 10). For other conformations, the signs of these two kinds of contributions may vary independently. Usually, the exciton CD contribution will be relatively small, since the ^1L_b tyrosyl absorption bands are not intense enough to give a large splitting between the positive and negative exciton branches. Mainly, the exciton contribution serves to enhance the vibronic structure in tyrosyl CD spectra, even at room temperature, e.g., see Figure 2 in Reference 52 and Figures 2 and 3 in Reference 50.

Damle[82] has suggested that the near-UV CD band of poly-Tyr in trimethylphosphate is an

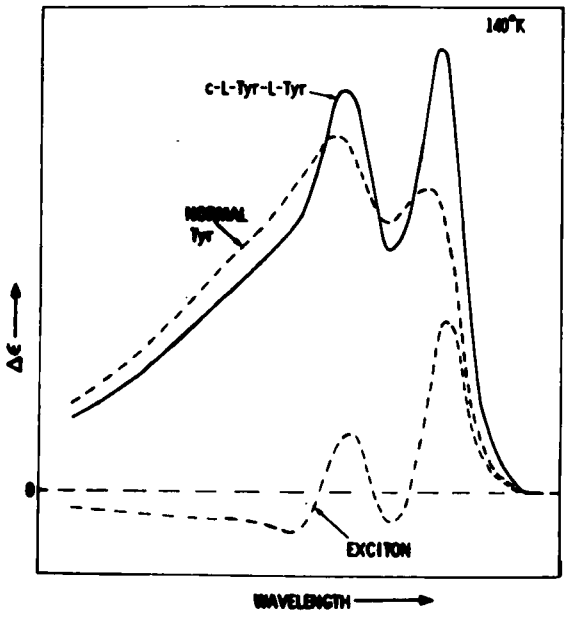

FIGURE 10. Approximate resolution of CD spectrum of *cyclo*(-Tyr-Tyr-) into normal and exciton components. (From Strickland et al.,[52] *J. Biol. Chem.*, 245, 4174, 1970. With permission.)

exciton band. Although this CD spectrum does show a sign reversal at about 275 nm, it is also consistent with multiple side chain conformers being present. Woody and his co-workers have presented both theoretical[41] and experimental[83] evidence that a variety of side chain conformers may exist in poly-Tyr. Similarly, the sign reversal in the near-UV tyrosyl CD band[84] of the helical polypeptide (-Tyr-Ala-Glu-)$_{200}$ may possibly result from multiple side chain conformers having different CD signs.

Range of CD intensities for tyrosyl model compounds – For the *N*-acyl esters and amides of tyrosyl and *O'*-methyltyrosyl, the $\Delta\epsilon$ values range from +0.2 to −0.2 $M^{-1}\,cm^{-1}$ in various polar solvents at room temperature (see Figure 9 and Table 3[85-90]). When these derivatives are dissolved in methylcyclohexane, however, $\Delta\epsilon$ increases to 0.6 to 0.8 $M^{-1}\,cm^{-1}$ at 297°K (see Figure 8 and Table 3). Perhaps the enhanced CD in this nonpolar solvent results because a single tyrosyl conformation is stabilized by strong interactions between the polar portions of the amino acid moiety and the aromatic ring.[74] In the case of diketopiperazines, the CD intensities are much less influenced by solvent changes and are larger ($\Delta\epsilon_{Tyr}$, 0.7 to 1.4 $M^{-1}\,cm^{-1}$ at room temperature). Some enhancement of rotatory strength occurs for the diketopiperazines when cooled to 140°K.[52] At 140°K, where the side chains should be more rigid, the rotatory strength per tyrosyl is greatest in those diketopiperazines having two aromatic residues with the L-configuration, i.e., *cyclo*(-Phe-Tyr-) and *cyclo*(-Tyr-Tyr-) in Table 3. Their $\Delta\epsilon$ to ϵ ratio is about 1.1 x 10^{-3} at 140°K.

Interestingly, the CD bands of numerous cyclic hexapeptides and polymers (see Table 3) are relatively weak ($|\Delta\epsilon^{max}_{Tyr}|$ less than $0.4M^{-1}_{Tyr}\,cm^{-1}$). Possibly, these small CD intensities result because their tyrosyl side chains are relatively motile, even though their peptide backbones are fairly rigid. Alternatively, their tyrosyl side chains may be rigid but have a conformation giving a weak CD band.

Mechanisms generating tyrosyl CD – Some years ago, Moscowitz et al.[91] described how the one-electron mechanism might possibly contribute to the ^1L_b tyrosyl CD band. Their suggestion was based partly on theoretical intuition and partly on extremely limited data, namely, that the rotatory strength of L-phenylalanine in water was much less than that of L-tyrosine in water even after accounting for their difference in absorption intensity. Since these compounds may have different conformations in water, however, comparing a single data set is not conclusive. Examining the rotatory strength to dipole strength ratios for the most intense CD spectra reveals values of about 1 to 2 x 10^{-4} for both tyrosyl and phenylalanyl compounds (calculations based upon data cited in Tables 2 and 3). This result is consistent with the hypothesis that nondegenerate μ–μ coupling is a major source of near-UV CD for both tyrosyl and phenylalanyl.

Recently, Hooker and Schellman[19] have considered in more detail which mechanisms may contribute to the ^1L_b CD bands of L-tyrosine. Their calculations suggested that coupling of the ^1L_b tyrosyl band with the far-UV carboxylate π–π^* transition may be the main source of CD for L-tyrosine. Similarly, Chen and Woody[41] found that coupling of the ^1L_b tyrosyl band with the far-UV electronic transitions of poly-Tyr gives rotatory strengths within the range observed experimentally for various solvents.

Thus, the available evidence strongly suggests that μ–μ coupling may be the major source of ^1L_b tyrosyl CD. If the geometry is optimum, a large ^1L_b tyrosyl CD band may result from coupling

TABLE 3

Near-UVCD Intensities of Selected Tyrosyl Model Compounds at Room Temperature, unless Otherwise Indicated

Compound[a]	Solvent	$\Delta\epsilon_{Tyr}^{max}$[b]	λ^{max}, nm	Reference
Tyr	H_2O (pH 1)	0.4	274	4, 19, 85
Ac-Tyr-NH_2	H_2O	−0.14	275	49, 66, 86
	8 *M* guanidine-HCl	−0.15	275	76
Ac-Tyr-OEt	dioxane	0.20	277	49
N-stearyl-Tyr *n*-hexyl ester	CH_3OH	−0.13	278	49
	C_7H_{14}	0.63	277	74
cyclo(-Gly-Tyr-)	dioxane	0.67	278	50
	H_2O	0.83	274	50
	EPA	0.96	279	52
cyclo(-Tyr-Tyr-)	dioxane	0.97	285	50
	H_2O	0.89	275	50
	EPA	1.35	279	52
cyclo(-Leu-Tyr(Me)-)	CF_3CH_2OH	1.2	273	51
cyclo(-Phe-Tyr-)	EPA	1.3	279	52
cyclo(-Gly_5-Tyr-)	H_2O	−0.2	278	87
cyclo(-Gly_2-Tyr-Gly_2-His-)	H_2O	−0.2	275	87
cyclo(-Gly-His-Gly--Ala-Tyr-Gly-)	H_2O	−0.16	275	81
cyclo(-Gly-His-Gly--Tyr-Ala-Gly-)	H_2O	0.16	270	81
cyclo(-Gly_2-Tyr-Gly_2-Tyr-)	H_2O	−0.27	275	81
(-Tyr-Ala-Glu-)$_{200}$ (helical)	c	−0.33	273	84
copolymer of Glu and Tyr (23:1) (helical)	d	−0.35	276	88
copolymer of N^5-(2-hydroxyethyl)-Gln and Tyr (17 mol %) (helical)	H_2O-CH_3OH (8:2)	−0.29	277	89
poly-Tyr (helical)	$(CH_3O)_3PO$	−0.3	283	82
poly-Tyr (random coil?)	CH_3 OH + 6*M* LiCl	−0.3	276	90
cyclo(-Gly-Tyr-)	EPA (140°K)	1.5	279	52
cyclo(-Val-Tyr-)	EPA (140°K)	1.6	279	52
cyclo(-Phe-Tyr-)	EPA (140°K)	2.3	279	52
cyclo(-Tyr-Tyr-)	EPA (140°K)	2.5	286	52

[a] Abbreviations according to Reference 60.
[b] $\Delta\epsilon_{Tyr}^{max}$, maximal $\Delta\epsilon$ value per tyrosyl in units of $M_{Tyr}^{-1}cm^{-1}$.
[c] 0.15 *M* NaCl-0.02 *M* sodium phosphate, pH 7.4.
[d] Water-dioxane (2:1, v:v) with 0.2 *M* NaCl, pH 3.8.

with the far-UV transitions of neighboring aromatic side chains (Tyr, Phe, Trp, His), the peptide $\pi-\pi^*$ transitions, and perhaps the ^1L_a tryptophanyl transition. The suggested importance of these coupling interactions is consistent with the large rotatory strengths observed for the diketopiperazines having stacked aromatic rings.[52]

The possible CD contributions arising from some types of $\mu-m$ couplings have not received detailed consideration. Coupling of the ^1L_b tyrosyl band with a magnetic dipole transition of a nearby disulfide group may possibly give significant rotatory strength, if the geometry is favorable.[92]

Near-UV Tryptophanyl CD Bands

This spectral characterization has been unusually difficult, because both the ^1L_a and ^1L_b electronic transitions overlap extensively in the near-UV region. Fortunately, though, each of these transitions is intense enough that many of its CD characteristics may be predicted from the

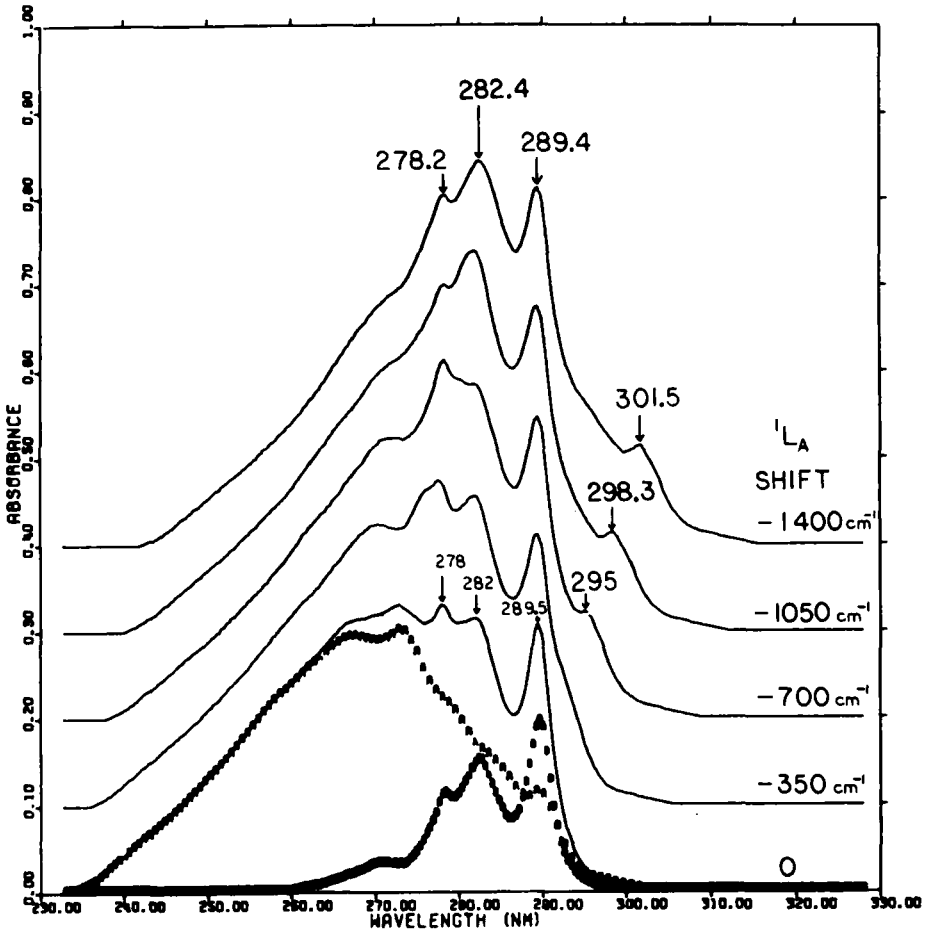

FIGURE 11. Effects of shifting ^1L_a band upon the shape of the tryptophanyl absorption spectrum. Bottom trace (–) is the spectrum of *N*-stearyl tryptophan *n*-hexyl ester dissolved in methylcyclohexane at 297°K;[93] AAA, ^1L_a component; BBB, ^1L_b component. Upper spectra indicate shapes observed after red shifting the ^1L_a band relative to the ^1L_b band. For ^1L_b band, $\mu \approx 1.2 \times 10^{-18}$ cgs; for ^1L_a band, $\mu \approx 2.6 \times 10^{-18}$ cgs.

properties of its corresponding absorption band, i.e., nontotally symmetrical vibrations would not be expected to contribute significantly to either the absorption or CD spectra. If a single transition of each tryptophanyl species is considered separately, its CD band should have about the same shape and wavelength position as its absorption band, when it can be resolved.

Vibronic transitions – Studies of 5-methoxyindole spectra have permitted a reliable resolution of the ^1L_a and ^1L_b bands of *N*-stearyl-L-tryptophan *n*-hexyl ester dissolved in methylcyclohexane (see Figure 11),[93] which gives a spectrum corresponding to a tryptophanyl side chain buried in a nonpolar region of a protein. Since environmental perturbations can shift the ^1L_a band relative to the ^1L_b band, the tryptophanyl absorption band may also have somewhat different shapes. Figure 11 shows the effects of shifting the ^1L_a band to longer wavelengths by amounts comparable to those observed in model compounds and proteins.[78,94] An important result from these spectra is that the vibronic structure observed from 275 to 290 nm arises primarily from the ^1L_b bands even though the broader ^1L_a bands may contribute more of the total intensity (see Figure 11). To obtain spectra resembling those observed for proteins at room temperature, the vibronic bands would need to be broadened somewhat, since the interactions giving wavelength shifts also tend to blur the spectra.

In some ways, the tryptophanyl ^1L_b bands

resemble the tyrosyl ^1L_b bands. Both have about the same dipole strength and are characterized by two prominent vibronic bands, 0–0 and 0 + 850 cm^{-1} for tryptophan[24,95] vs. 0–0 and 0 + 800 cm^{-1} for tyrosine.[49] In contrast to tyrosyl, the tryptophanyl 0–0 ^1L_b band is more intense than any of the other ^1L_b vibronic bands (see Figure 11), and its wavelength position is only slightly red shifted by hydrogen bonding its side chain (0 to 1.5 nm).[78] Interactions with nearby polar groups and changes in polarizability of the surroundings may also shift the tryptophanyl ^1L_b band by a few nm. Usually, the 0–0 ^1L_b band of tryptophan lies between 288 and 292 nm, although it may be shifted as far as 287 or 293 nm in some proteins.[94]

The characteristics of the tryptophanyl ^1L_a transition differ greatly from those of the ^1L_b transition. The ^1L_a dipole strength is about four times larger than that of the tryptophanyl ^1L_b band.[93] Under most experimental conditions, the ^1L_a transition lacks any obvious vibronic structure. The ^1L_a band usually overlaps both the long- and short-wavelength ends of the ^1L_b tryptophanyl band. Most of the ^1L_a intensity occurs at the short-wavelength side of the ^1L_b band (see Figure 11), although the long-wavelength ^1L_a bands are sometimes quite evident in CD spectra at room temperature[24] or in absorption spectra of proteins at 77°K.[94] Molecular orbital calculations by Song and Kurtin[96] indicated that the ^1L_a excited state has a much larger permanent dipole moment than either the ^1L_b or the ground state. Thus, electrostatic interactions with polar groups close by the indolyl ring of a protein may cause appreciable shifts to either longer or shorter wavelengths, depending upon the geometry.[9,94] Similarly, the presence of highly polarizable groups* surrounding the indolyl ring may cause the ^1L_a band to red shift. Hydrogen bonding the indolyl >NH may possibly cause red shifts of up to 12 nm, depending upon which acceptor group is involved.[78] The largest ^1L_a red shifts are expected for hydrogen bonding to carboxylate ions, to the $>\ddot{N}$ of histidyl side chains, or to the carbonyl oxygens of the peptide backbone and side-chain amides. Apparently, the wavelength position of the 0–0 ^1L_a tryptophanyl band may range from about 289 to 302 nm.

Transition moment directions of ^1L_a and ^1L_b bands – Disagreement exists over the correct directions; e.g., compare predictions from Reference 97 with those from Reference 98. Until these directions are firmly established, theoretical calculations of tryptophanyl CD intensities will remain suspect.

Artifacts in CD spectra of tryptophanyl compounds – For many tryptophanyl compounds, the ratio of CD to absorption is unusually small ($\Delta\epsilon/\epsilon \leqslant 3 \times 10^{-5}$). Excellent instrument performance is required to prove the existence of vibronic bands and to avoid absorption-related artifacts (see *CD Artifacts* in section on *Experimental Aspects of Near-UV CD Measurements*). In the case of noisy spectra, the instrument noise may be misinterpreted as a vibronic CD band. Identifying weak vibronic CD bands is greatly facilitated by signal averaging.[18] Unfortunately, though, artifactual signals can also be built up into an apparent CD band by averaging many spectra. In this regard, Myer and MacDonald[17] reported extensive vibronic structure in the CD spectrum of L-tryptophan in water relative to a water base line. Strickland et al.[24] were unable to confirm the existence of these vibronic bands in comparing L- and D-tryptophan in water relative to a D,L-tryptophan and a water base line (see Figure 12). Since their instrument was capable of resolving complex vibronic structure in phenylalanyl compounds,[25] it had sufficient resolution to observe vibronic bands for L-tryptophan in water, if any existed. Apparently, Myer's and MacDonald's instrument may have generated an absorption-related artifact, although this conjecture cannot be proved because they did not compare spectrum of nonoptically active compound with that of solvent.

Similarly, Strickland and Billups[76] have been unable to observe the sharp vibronic bands reported by Shiraki[66] for Ac-Trp-NH_2 dissolved in water. Especially, we do not observe either a negative CD band on the short-wavelength side (266 nm) or a vibronic band at 271 nm.

CD spectra of compounds containing only one tryptophanyl – A variety of CD spectra are observed for tryptophanyl, because there are two near-UV electronic transitions whose CD signs and intensities may vary independently. These spectra have been classified into four types.[24]

Type 1: The ^1L_b electronic transition has strong CD bands, whereas the ^1L_a bands have little or no obvious CD intensity. These CD spectra are

*e.g., aromatic side chains.

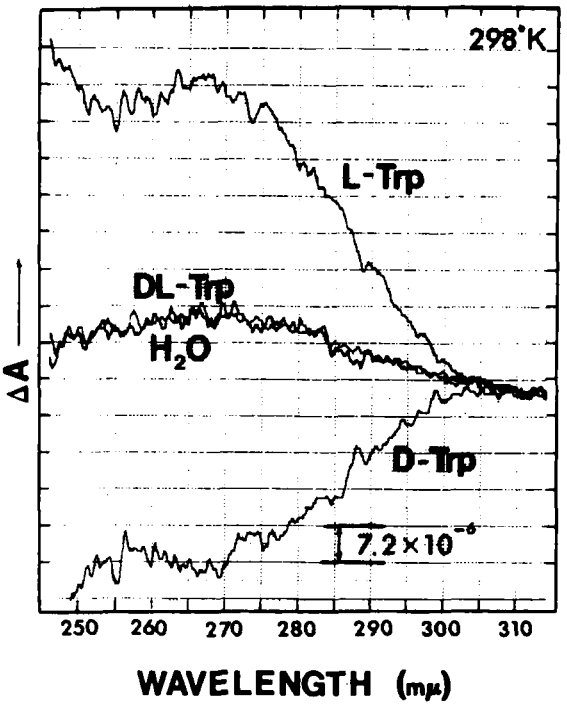

FIGURE 12. Example of a ^1L_a-type tryptophanyl CD spectrum. CD records of 0.14 m*M* L-Trp, D-Trp, and DL-Trp in neutral water at 298°K. Both DL-Trp (dark trace) and H_2O (faint trace) gave the same base line. Path length, 10 mm. (Reprinted from Strickland et al.,[24] *Biochemistry*, 8, 3209, 1969, © 1969 by the American Chemical Society. Reprinted by the permission of the copyright owner.)

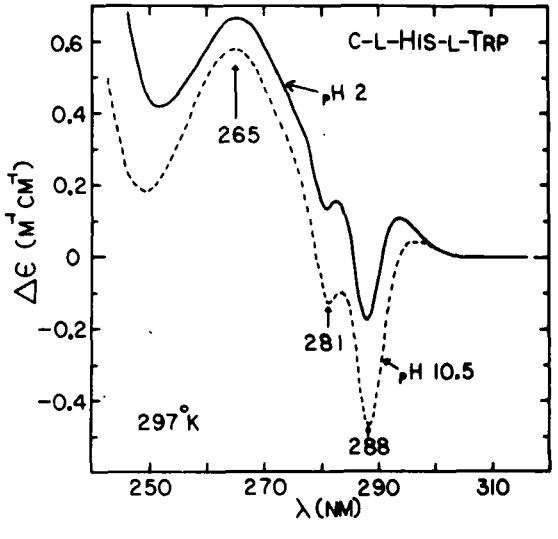

FIGURE 14. Example of tryptophanyl CD spectrum having both ^1L_a and ^1L_b CD bands. *cyclo*(-His-Trp-) dissolved in water at pH 2 (–) and at pH 10.5 (- - -). (From Strickland, Wilchek, and Billups.[59])

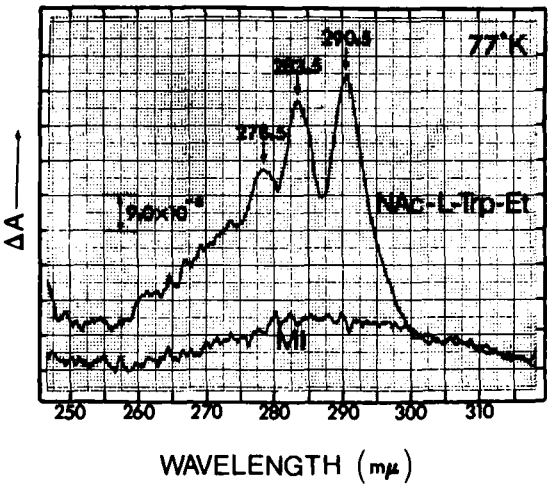

FIGURE 13. Example of a ^1L_b-type tryptophanyl CD spectrum. CD record of Ac-Trp-OEt (3.8 m*M*) in EPA at 77°K. The base line (MI) is 3-methylindole having the same absorbance as Ac-Trp-OEt. Path length, 0.2 mm. (Reprinted from Strickland et al.,[24] *Biochemistry*, 8, 3208, 1969, © 1969 by the American Chemical Society. Reprinted by permission of the copyright owner.)

dominated by two vibronic CD bands (0–0 and 0 + 850 cm^{-1}) having the same sign which may be either positive or negative. To a first approximation, these CD spectra (see Figure 13) have shapes similar to those shown for the resolved ^1L_b absorption band (see Figure 11), except that the band widths are broadened in water and other polar environments.

Type 2: These CD spectra (see Figure 12) arise mainly from the ^1L_a electronic transition. They lack any major vibronic structure and have shapes resembling the resolved ^1L_a absorption band shown in Figure 11.

Type 3: Both the ^1L_a and ^1L_b electronic transitions have obvious CD bands. If these spectra are resolved by curve analysis, each electronic transition (^1L_a or ^1L_b) has a CD envelope (either positive or negative) having about the same shape as the corresponding absorption envelope. *cyclo*(-His-Trp-) in water (see Figure 14) illustrates a type 3 spectrum having a positive ^1L_a CD band and negative ^1L_b vibronic bands (288 and 281 nm). Note that at pH 2, the negative ^1L_b vibronic band at 281 nm is raised into the positive region by the overlapping broad positive ^1L_a CD band that extends from about 305 to 250 nm. In spectra having opposite signed CD bands, knowledge of the ^1L_b vibronic spacing and the wavelength of

the 0–0 band is often necessary to determine if one has positive vibronic bands (e.g., at 283.5 nm in Figure 14) or negative vibronic bands (e.g., at 281). Please observe also that the apparent positive CD band at about 295 nm is a pseudoband caused by superposition of the narrower ^1L_b bands on top of the broad ^1L_a band. In other words, the positive CD at 295 nm does not represent a ^1L_a vibronic transition. Thus, Figure 14 illustrates clearly that a knowledge of absorption spectra facilitates identifying CD bands.

Type 4: The wavelengths of CD and absorption bands have a large mismatch, either due to multiple species or due to overlap from the far-UV tryptophanyl band. One major subgroup has a CD plateau extending from about 270 to 255 nm, e.g., *cyclo*(-Gly-Trp-) dissolved in water (Figure 4 in Reference 50) or in methanol-glycerol at 297°K (Figure 4 in Reference 52). If two tryptophanyl species have opposite ^1L_a signs, the ^1L_a band becomes weaker and may undergo an inversion in CD sign between the long- and short-wavelength ends, as has been observed for multiple species of tyrosyl (see *Near-UV Tyrosyl CD Bands*). In the case of the tryptophanyl ^1L_b transition, the existence of multiple species may shift both vibronic CD bands by 1 or 2 nm from the wavelengths of their absorption bands. If two species have opposite CD signs with the ^1L_b band differentially shifted, the major ^1L_b vibronic CD bands become sharper, even though they are weaker.

CD spectra of compounds containing two or more tryptophanyls – Just as with tyrosyl bands, the appearance of these spectra depends on whether all sites are rigid and identical. If not, these spectra may resemble those described for multiple species of compounds having only one tryptophanyl.

The potential for tryptophanyl exciton bands may be judged in terms of the dipole strengths of the two transitions. On this basis, one expects that the ^1L_b tryptophanyl band may give small exciton CD bands similar to those of tyrosyl, and that the ^1L_a band may show appreciably more exciton CD, if the geometry is favorable. Two spectra have been reported that are consistent with having an exciton CD contribution superimposed upon single species-type bands: ^1L_a exciton for $(\gamma\text{-Et-DL-Glu})_{116}$ $(\text{L-Trp})_{32}$ in trifluoroethanol (Figure 4 in Reference 99) and both ^1L_a and ^1L_b exciton for *cyclo*(-Trp-Trp-) in EPA at 140°K (Figure 7 in Reference 52).

Range of CD intensities for tryptophanyl model compounds – The CD intensities of low-molecular weight tryptophanyl compounds can be affected by solvent changes and can be enhanced appreciably by cooling. At room temperature, the absolute value of $\Delta\epsilon_{Trp}$ is less than $0.4\,M_{Trp}^{-1}$ cm^{-1} for tryptophan, Ac-Trp-OEt, Ac-Trp-NH_2, and linear dipeptides (Table 4[100] and Reference 50). Many of the cyclic dipeptides have much more intense CD bands ($|\Delta\epsilon_{Trp}|$ ranges up to $1.7\,M_{Trp}^{-1}$ cm^{-1}). At 140°K, the $\Delta\epsilon_{Trp}$ values are largest for diketopiperazines having another aromatic ring adjacent to the indolyl ring (*cyclo*(-His-Trp-), *cyclo*(-Phe-Trp-), *cyclo*(-Trp-Trp-)) (Table 4, Reference 52).

A similar intensification has been observed by Peggion et al.[100] in random copolymers containing an increased ratio of L-tryptophan relative to γ-ethyl-L-glutamate (see Table 4). At 32% tryptophan, the $\Delta\epsilon_{Trp}^{max}$ value is only about $0.1\,M_{Trp}^{-1}$ cm^{-1}. By 86% tryptophan, the value has increased to $1\,M_{Trp}^{-1}$ cm^{-1}. Poly-Trp and a block copolymer have even more intense CD spectra ($\Delta\epsilon_{Trp}$ = 1.6 and $2.6\,M_{Trp}^{-1}$ cm^{-1}, respectively). These findings suggest that interactions between aromatic side chains may give enhanced tryptophanyl CD intensities.[52,100]

Interestingly, the largest $\Delta\epsilon$ values in Table 4 are about $2.5\,M_{Trp}^{-1}$ cm^{-1}, both in a compound having a ^1L_a band as the most intense band (block copolymer) and in a compound having a ^1L_b band as its most intense band (*cyclo*(-Trp-Trp-) at 140°K). For the ^1L_b tryptophanyl band, the largest $|\Delta\epsilon|$ to ϵ ratio is comparable to that observed for tyrosyl and phenylalanyl bands (about 1×10^{-3}). In the case of the ^1L_a tryptophanyl band, however, this ratio is lower (about 0.5×10^{-3}). Perhaps the apparent weakness of the ^1L_a tryptophanyl CD band is related in part to three factors: (A) the limited number of compounds studied, especially at low temperatures, (B) overlap of the ^1L_a maximum by the short wavelength side of the ^1L_b band, and (C) cancellation from multiple species having opposite CD signs.

Mechanisms generating tryptophanyl CD bands – Based upon the dipole strengths of the ^1L_a and ^1L_b bands (see Figure 11), μ–μ coupling may be expected to be a major source of tryptophanyl near-UV CD. The importance of this mechanism is

TABLE 4

CD Intensities of Selected Tryptophanyl Model Compounds

Compound[a]	Solvent	T[b], °K	^1L_b Region[c] $\Delta\epsilon_{Trp}^{max}$	^1L_b Region[c] λ^{max}, nm	^1L_a Region[c] $\Delta\epsilon_{Trp}^{max}$	^1L_a Region[c] λ^{max}, nm	Reference
Trp	H_2O (pH 7)	298			0.34	266	24, 85
Ac-Trp-NH_2	H_2O (pH 7)	297	0.2	288			76
	8 *M* guanidine-HCl	297	0.24	289	0.15	d	76
Ac-Trp-OEt	EPA	297	0.06	291	e		52
	EPA	140	0.6	291			52
N-stearyl-Trp *n*-hexyl ester	C_7H_{14}	297	−0.10	289	0.27	253[f]	59
cyclo(-His-D-Trp-)	H_2O (pH 2)	297			−0.80	270	59
cyclo(-Gly-Trp)	H_2O	RT			1.1	266	50
	$(CH_3)_2SO$	RT	0.94	291			50
	dioxane	RT	0.27	284	−0.1	264	50
	EPA	140	0.70	291			52
cyclo(-D-Val-Trp-)	EPA	140			−0.69	267	52
cyclo(-His-Trp-)	EPA	297	−0.72	290			59
	EPA	140	−1.8	291	−0.8	268[g]	59
cyclo(-Phe-Trp)	EPA	297	−1.4	290			52
	EPA	140	−2.2	290	−0.9	270[g]	52
cyclo(-Trp-Trp-)	H_2O	RT	−1.1	289	0.3	269	50
	$(CH_3)_2SO$	RT	−0.52	292	0.14	300	50
	EPA	297	−1.7	290	−0.90	260	52
	EPA	140	−2.4	291	−1.4	265	52
copolymer of Trp and Glu (OEt)							
32 mol %-Trp-	$CH_3OCH_2CH_2OH$	RT			0.1	275	100
86 mol %-Trp-	$CH_3OCH_2CH_2OH$	RT	0.8	292	1.0	268	100
poly-Trp (helical)	$CH_3OCH_2CH_2OH$	RT	1.3	292	1.6	266	100
Block copolymer of $[(\text{-DL-Glu(OEt)-})_{116}(\text{-Trp-})_{32}]$	CF_3CH_2OH	RT			2.6	272	99

[a] Abbreviations according to Reference 60.
[b] T, temperature; RT, room temperature.
[c] Spectra were divided into ^1L_a and ^1L_b regions. No attempt was made to further separate ^1L_a and ^1L_b contributions. $\Delta\epsilon_{Trp}^{max}$, maximal $\Delta\epsilon$ value per tryptophanyl in units of $M_{Trp}^{-1}cm^{-1}$.
[d] Plateau from 255 to 270 nm.
[e] Not measured.
[f] Narrow plateau.
[g] Broad shoulder.

supported by the increased CD intensity observed in compounds having another aromatic ring juxtaposed with the tryptophanyl side chain (see above).

OTHER GROUPS WHICH MAY HAVE NEAR–UV CD

In addition to the aromatic side chains, proteins may contain other moieties with near-UV CD bands. Among the naturally occurring amino acid residues, only the cystinyl side chains have been shown to have CD in this region. If a protein contains a prosthetic group having a near-UV absorption band, even a weak one, it also may have near-UV CD bands. These bands should be thoroughly characterized before attempting to identify the aromatic amino acid CD bands in conjugated proteins. This section briefly summarizes the CD properties of cystinyl side chains, heme, and certain bound cations, because they

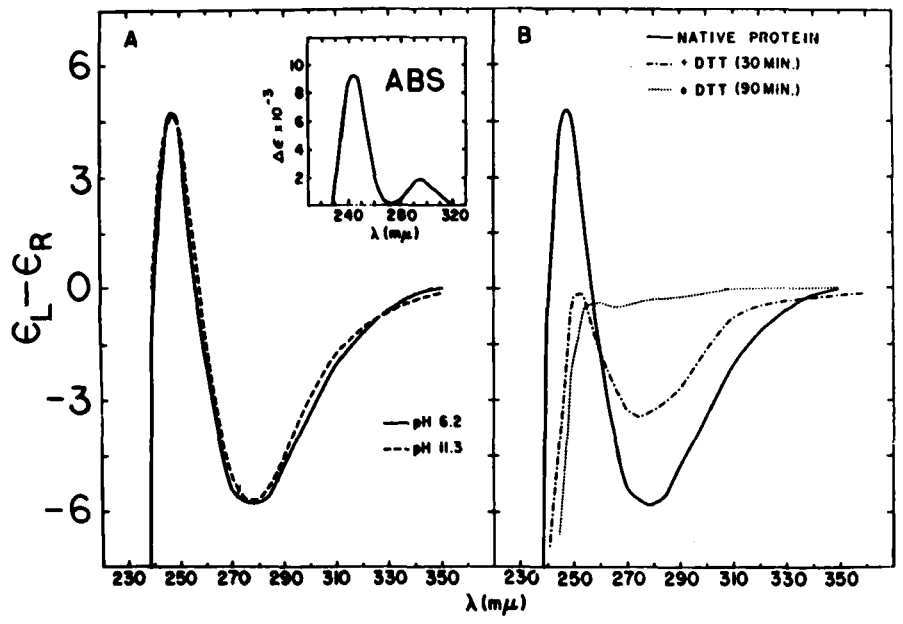

FIGURE 15. Left: CD spectra of neurophysin-II at pH 6.2 and 11.3. The insert (ABS) shows the change in *absorption* upon raising the pH from 6.2 to 11.3. Right: CD spectrum of neurophysin-II at pH 6.2 before and after adding 20 m*M* dithiothreitol (DTT). All spectra at room temperature. Native protein has seven disulfides, one Tyr, three Phe, and no Trp.[107] (From Breslow, *Proc. Natl. Acad. Sci. U.S.A.*, 67, 495, 1970. With permission.)

occur in some of the proteins described in this review.

Cystinyl Side Chain

Although the near-UV absorption band of disulfides is very weak, its CD may be strong.[2] Apparently, its CD intensity may be determined by three factors: the dihedral angle of the disulfide,[101-104] the C–S–S bond angle,[2,22] and vicinal interactions.[105] In addition, the wavelength positions of these bands are affected by the dihedral angle.[101,103,104] Thus, the shapes and intensities of the disulfide CD bands are highly conformation-dependent and have not been well-characterized.

Usually, the disulfide CD of cystinyl begins at long wavelengths (320 to 350 nm) and gradually intensifies to give one or two broad bands located above 240 nm. For example, neurophysin-II has a negative disulfide band at 277 nm and a positive band at 248 nm (see Figure 15.)[106,107] Disulfide CD bands are much broader than the CD bands observed for the aromatic amino acid side chains and lack fine structure even at 77°K.[49] Within the range of dihedral angles usually observed for cystinyl side chains in proteins (65 to 115°),[108] the longest-wavelength disulfide CD band probably can be expected to peak below 290 nm (see Figure 4 in Reference 103). In both cystinyl compounds and two model proteins, the first disulfide CD band occurs between 280 and 250 nm (see Table 5).[109-112] The disulfide CD of neurophysins and of lima bean trypsin inhibitor averages almost -1 $M^{-1}\,cm^{-1}$ per disulfide bond, which is the value reported for L-cystine cooled to 203°K (see Table 5). Possibly even more intense disulfide CD may occur in some proteins, because a rigid disulfide compound with a dihedral angle of about -60° has a $\Delta\epsilon^{max}$ of $-5\,M^{-1}\,cm^{-1}$ at 290 nm.[105,113]

In the proteins presently known to have intense disulfide CD (neurophysins-I and-II,[106,107] lima bean trypsin inhibitor,[112] human serum albumin,[114] insulin,[2] ovomucoid,[115] ribonuclease,[49] and ovine luteinizing hormone),[116] the longest wavelength disulfide CD band has been negative. Perhaps also in other proteins having intense disulfide CD, the long wavelength band will tend to be negative. This condition, however, is not likely to be universal, because positive disulfide CD has been predicted theoretically for certain dihedral angles[101-104] and has been observed for L-cystinyl derivatives, or expected from

TABLE 5

Longest Wavelength Disulfide CD Band of Selected Cystinyl Compounds and Model Proteins at Room Temperature, unless Otherwise Indicated

Compound	Solvent	λ^{max}, nm	$\Delta\epsilon^{max}_{-S-S-}$[a]	Reference
L-cystine	H_2O	257	−0.7	105, 109
	0.1 *M* NaOH	270	−0.2	105, 85
	(293°K)[b]	255	−0.4	110
	(203°K)[b]	255	−1.0	110
L-cystine diethyl ester·2HCl	CH_3OH	251	−0.4	105
	$(CH_3)_2CHOH$	255	0.3	105
Cys-Ile-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	H_2O (pH 2)	280	−0.1	111
cyclo(-D-Cys-Val-D-Cys-D-Leu-Ile-)[c]	CF_3CH_2OH	280	−1.2	105
cyclo(-Cys-Leu-D-Phe-Pro-Val-Cys-Leu-D-Phe-Pro-Val-)[d]	C_2H_5OH	272	−2.3	102
neurophysins-I and-II[e]	pH 6	280	−0.9	106, 107
acetylated lima bean trypsin inhibitor[e]	pH 7.5	280	−0.9	112

[a] $\Delta\epsilon^{max}_{-S-S-}$, maximal $\Delta\epsilon$ value per disulfide bond in units of $M^{-1}_{-S-S-}cm^{-1}$.
[b] 0.1 *M* HCl in 90% ethanol.
[c] Conformation I of malformin-A.
[d] Disulfide dihedral angle about 120°.
[e] Averaged over seven disulfide bonds.

the mirror image relationship with a D-cystinyl peptide (see Table 5).

Heme

The CD bands of protein-bound heme have been examined, using as a model the heme undecapeptide of cytochrome c, which lacks aromatic amino acid residues and disulfide bonds.[117] Although heme CD extends throughout the near-UV in both the ferro and ferri forms, none of these bands possesses fine structure,[117] even at 77°K.[118]

Bound Cations

Ions lacking near-UV absorption, e.g., Na^+, K^+, Ca^{++}, will not themselves have near-UV CD when bound to a protein. Apparently, Cu^{++} sometimes has strong near-UV CD bands[119-120] and sometimes does not.[121] Co^{++},[122] Cd^{++},[122,123] and probably Hg^{++} (see Figure 3 in Reference 124) may have relatively intense near-UV CD when bound to a protein. In some cases, removal of Zn^{++} from a protein does not alter its near-UV CD spectrum.[125,126] Zinc-histidine complexes do not have a Cotton effect above 240 nm.[127] On the other hand, removing Zn^{++} from the regulatory subunit of aspartate transcarbamylase abolishes a very intense CD band at 252 nm[128] and reduces the absorption intensity below 270 nm.[124] Probably the type of ligand influences the wavelength position of the Zn^{++}-ligand CD band. A Zn^{++}-mercaptide linkage may give the longest wavelength CD band.

AROMATIC CD BANDS IN PROTEINS

Intensities

Interpreting CD spectra may be facilitated by knowing the range of CD intensities anticipated for a single aromatic side chain in proteins (see Table 6). From a qualitative viewpoint, three factors influence the intensities of aromatic CD bands: rigidity of protein, interactions of aromatic ring with its surroundings, and number of aromatic residues. The studies on model compounds indicate that motility of the side chains tends to decrease CD intensity.

Both experimental and theoretical considerations indicate that some interactions are more likely to cause intense CD than others. When a tyrosyl or tryptophanyl side chain is less than 10 Å from another aromatic side chain (His, Tyr, Trp,

TABLE 6

Anticipated Range of $\Delta\epsilon^{max}$ Values for a Single Aromatic Side Chain of Proteins at Room Temperature[a]

Side Chain	$\Delta\epsilon^{max}$ (M^{-1} cm^{-1})
Phe (^1L_b)	± 0.3
Tyr (^1L_b)	± 2.0
Trp (^1L_b)	± 2.5
Trp (^1L_a)	± 3[b]

[a]Data based upon model compounds at 297°K and at 77°K with correction applied to give bandwidths equivalent to those at 297°K.

[b]May be as large as ±5 in most favorable site, if the largest $\Delta\epsilon/\epsilon$ is the same for ^1L_a Trp as that observed for other aromatic bands (1×10^{-3}).

Phe), the near-UV CD may be especially intense due to μ–μ coupling. Coupling between the near-UV transition of a tyrosyl or tryptophanyl side chain and the π–π^* transitions of peptide bonds within about 8 Å may also give appreciable CD intensity. All of these interactions tend to be greatest when the groups are nearly in contact. One or two strong interactions are more likely to give intense CD than are many interactions with the same aromatic ring. Since some interactions may give positive CD and others may give negative CD, summing many different interactions tends to cause extensive cancellation.

Similarly, proteins having large numbers of aromatic side chains may not have intense CD bands due to cancellation by positive and negative contributions. Statistical considerations suggest that in the native state the average CD per residue will tend to decrease as the number of aromatic residues increases.[67] A simple example of this cancellation is provided by aspartate transcarbamylase. Combining its regulatory subunits (negative CD) and catalytic subunits (positive CD) causes extensive loss of CD above 270 nm.[129]

Characteristic Wavelength Profiles

Each of the four types of side chains having CD bands above 250 nm has certain distinctive features that may permit their identification. If a protein has a prosthetic group having near-UV CD bands, their characteristics must also be considered. The disulfide CD bands are broad and extend throughout the near-UV. Sometimes, these bands are most easily detected from their long wavelength tail extending above 310 nm. CD bands arising from aromatic residues may be superimposed upon a disulfide background, which is sometimes quite intense.

Tryptophanyls – When only one tryptophanyl is present in a protein, the 0–0 ^1L_b band may appear prominently in its CD spectrum, coinciding with its absorption band (288 to 293 nm). The 0 + 850 cm^{-1} ^1L_b tryptophanyl band, which is about 7 nm toward shorter wavelengths, should have the same sign and nearly as much intensity as the 0–0 band, after compensating for the background CD. Apoazurin (see Figure 16)[130] and azurin[121,131] provide an impressive illustration of positive ^1L_b tryptophanyl bands (292 and 285 nm) superimposed upon a negative CD background. The ^1L_a tryptophanyl CD bands may be difficult to identify in a protein having only one tryptophanyl, because they usually have little vibronic structure to distinguish them from a disulfide CD band.

In proteins having several tryptophanyls, extensive CD fine structure may occur between 290 and 305 nm, a region where only tryptophanyl has fine structure. Often, this fine structure is unusually sharp, because the overlapping bands have opposite CD signs and have their 0–0 bands at different wavelengths. When the most prominent tryptophanyl CD band is the sum of overlapping 0–0 ^1L_b bands from several side chains and perhaps also superimposed upon ^1L_a bands, it may not coincide with the band observed in the absorption spectrum. Nevertheless, a companion CD band (0 + 850 cm^{-1} ^1L_b) with the same sign should occur 6 to 8 nm on the short wavelength side. Such a pair of tryptophanyl bands is evident in the CD spectra of carboxypeptidase-A[28] and β-lactoglobulin.[132] CD fine structure involving the ^1L_a band is sometimes apparent in the region from 295 to 305 nm, e.g., in chymotrypsinogen-A,[24,133] chymotrypsin,[134] apo-and holo-glyceraldehydephosphate dehydrogenase,[135] and coat protein of tobacco mosaic virus.[136] Some analysis is needed, however, to identify the fine structure band at longest wavelength, because a tryptophanyl band superimposed upon a disulfide CD band of opposite sign may falsely suggest an extra tryptophanyl band.

Tyrosyl – If a protein has tryptophanyl fine structure, it may obscure the tyrosyl CD fine structure. In the absence of tryptophanyl CD, the

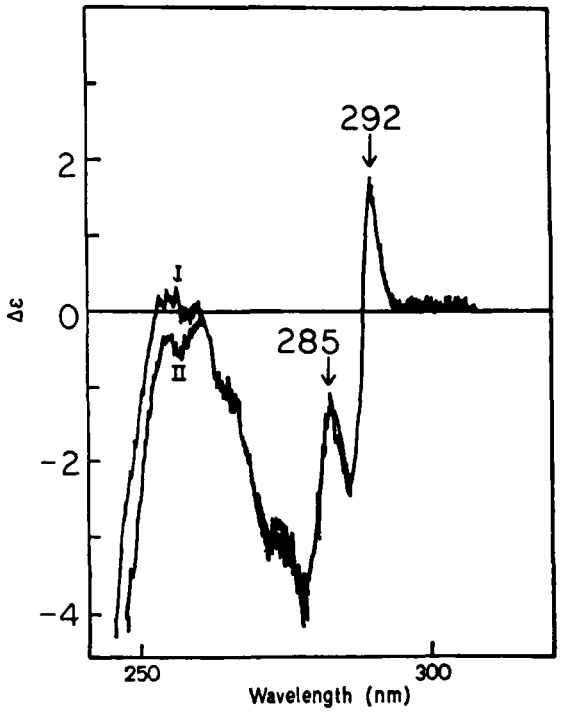

FIGURE 16. CD spectra of apoazurin (I) and reconstituted azurin (II) formed from adding $CuCl_2$ to apoazurin from *Pseudomonas fluorescens* at room temperature. Native protein has one Cu^{++}, one Trp, two Tyr, six Phe, and one disulfide bond. The wavelength of the 0–0 ^1L_b tryptophanyl CD band (292 nm) coincides with its wavelength in absorption[130] (A. Finazzi-Agro, personal communication). Published $\Delta\epsilon$ values[121] were corrected in this reproduced figure in accord with personal communication from A. Finazzi-Agro. (From Finazzi-Agro et al.,[121] *Eur. J. Biochem.*, 34, 22, 1973. With permission.)

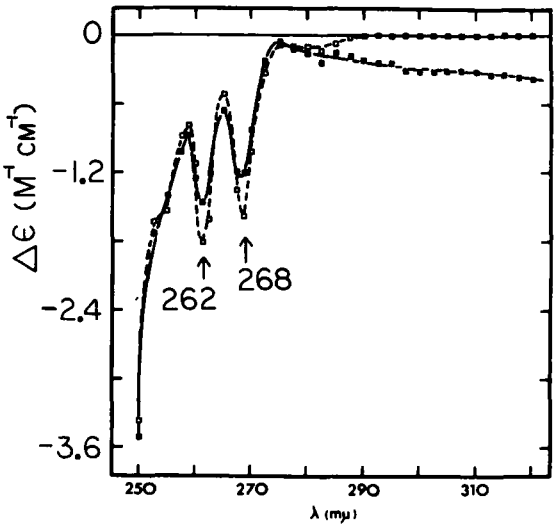

FIGURE 17. Negative phenylalanyl CD fine structure at 268 and 262 nm in troponin-*A* at room temperature. (■) in 50 m*M* Tris (pH 7.6) - 1 m*M* EGTA and (□) in 50 m*M* Tris (pH 7.6) - 5 x 10^{-4} *M* $CaCl_2$. Protein contains 0 Trp, 3 Tyr, 11 Phe,[141] and 0 disulfide per 22,200 molecular weight (C. M. Kay, personal communication). $\Delta\epsilon$ calculated relative to 22,200 molecular weight. Phenylalanyl fine structure $\Delta\epsilon^{268}$ is about −0.1 M_{Phe}^{-1} cm^{-1} per phenylalanyl in □ record, measured according to Reference 67. (Reprinted from Murray and Kay, *Biochemistry*, 11, 2625, 1972, © 1972 by the American Chemical Society. Reprinted by permission of the copyright owner.)

existence of tyrosyl CD may be revealed by its vibronic structure, superimposed upon the CD bands of other moieties. The tyrosyl CD maximum (about 0 + 800 cm^{-1}) is located between 275 and 282 nm. Its 0–0 band may be seen at room temperature as a shoulder located about 6 nm toward the red, e.g., see ribonuclease-A and -S,[49,137] insulin,[138,139] luteinizing hormone,[116] regulatory subunit of aspartate transcarbamylase,[129] and basic pancreatic trypsin inhibitor.[140]

Phenylalanyl – These side chains produce sharp CD fine structure between 270 and 255 nm, a region where other amino acid side chains give only broad bands. The sizes of the phenylalanyl bands can be approximated after correcting for the background CD.[67] Often, the phenylalanyl CD bands have different signs from the background CD, which hinders their identification unless one recalls three characteristics observed in model compounds. First, the 0–0 band (267-269 nm) usually has measurable CD and rises sharply from the background CD. Second, the prominent phenylalanyl CD bands occur in pairs separated by about 6 nm and having the same CD signs and nearly equal intensities. Third, the intensities of the various progressions usually differ and often have opposite CD signs. If multiple phenylalanyl sites (species) exist, the CD fine structure may be more complex than described.

Among the proteins studied, troponin-A (see Figure 17)[141] and two parvalbumins[142] are unusual in that their most prominent near-UV CD bands arise from phenylalanyl side chains. In many other proteins, the phenylalanyl fine structure is superimposed upon a more intense background CD, e.g., aspartokinase-homoserine dehydrogenase,[143] carboxypeptidase-A,[28] human chorionic somatomammotropin,[144] *Chironomus th. thummi I* hemoglobin,[145] fetuin,[146] fuma-

rase,[147] growth hormones,[148-151] immunoglobulin G and fragments,[152-154] ovalbumin,[133] apo horseradish peroxidase,[67] ribonuclease-A and -S,[137] serum albumins,[114,155] coat protein of tobacco mosaic virus,[136] and umecyanin.[156]

Identifying CD Bands by Modifying Side Chains

Modifying tyrosyl or tryptophanyl side chains has been widely used in attempts to identify the near-UV CD bands of native proteins. Unfortunately, many of these studies lacked both experimental rigor and a sound theoretical basis for interpreting the observed CD spectra. Four potential difficulties should be kept in mind when analyzing near-UV CD spectra of modified proteins: (A) The modification may alter the native conformation, either by changing the orientation of the modified aromatic side chain or by having unwanted reactions with nonaromatic side chains. (B) The reaction may not modify the side chain anticipated; e.g., "buried" tyrosyl sometimes reacts more easily than "exposed" tyrosyl.[157,158] (C) The modified side chain may have near-UV CD bands of its own. (D) Modifying a side chain may affect its interactions with neighboring aromatics or disulfide groups, causing their CD bands to be altered indirectly. Since μ–μ coupling may occur over some distance (up to about 10 Å), the probability of these interactions is appreciable.

Ionized tyrosyl side chain – The two lowest energy bands of the phenolate group are more intense and are located more to the red than the bands of unionized tyrosyl side chain.[159] The long wavelength phenolate band is polarized in the same direction as the ^1L_b tyrosyl band[160] ($D_s \approx 2.4 \times 10^{-36}$ cgs).

CD spectra of the phenolate group in model compounds have been studied by Legrand and Viennet,[85] by Beychok,[161] by Edelhoch, Lippoldt, and Wilchek,[50] and by Strickland, Wilchek, and Billups.[59] In compounds having a single phenolate group, its long wavelength CD band has about the same shape and wavelength maximum as its absorption band. The CD band of ionized *cyclo*(-Tyr-Tyr-), however, shows some shape distortion that is consistent with a small exciton contribution; e.g., the CD maximum is red shifted by 5 nm from the position of the absorption maximum (292 nm). The short wavelength phenolate CD band is sometimes quite intense and may extend as far as 275 to 280 nm, even though its absorption maximum is about 243 nm. The signs of these two phenolate CD bands may be opposite or may be the same (both positive or both negative).

TABLE 7

Intensities for Tyrosinate CD Bands of Model Compounds at Room Temperature (Mostly at pH 12.8)

Compound	$\Delta\epsilon^{max}_{Tyr}$[a]	λ^{max}, nm	Ref.
Tyr	0.35	293	85
Tyr-OEt	0.4	290	161
cyclo(-Gly-Tyr-)	0.9[b]	292	59
cyclo(-Val-Tyr-)	1.0	293	59
cyclo(-D-Trp-Tyr-)	0.4	299[c]	59
cyclo(-Trp-Tyr-)	1.7	297[c]	59
cyclo(-Phe-Tyr-)	1.9	293	59
cyclo(-Tyr-Tyr-)	2.0	297	50, 59

[a] $\Delta\epsilon^{max}_{Tyr}$, maximal $\Delta\epsilon$ value per tyrosinate in units of M^{-1} cm.$^{-1}$

[b] 0.5 M^{-1} cm^{-1} was reported by Edelhoch et al.[50]

[c] The small overlap from negative tryptophanyl CD bands causes the apparent CD maximum to be shifted to this longer wavelength.

Table 7 summarizes the long wavelength $\Delta\epsilon$ values for several tyrosinate model compounds at room temperatures. The cyclic dipeptides containing two aromatic amino acids with the L-configuration have especially large $\Delta\epsilon_{Tyr}$ values. Apparently, the presence of a second aromatic ring close by the phenolate ring causes appreciable enhancement of the phenolate CD intensity. The ratio of $\Delta\epsilon_{Tyr}$ to ϵ_{Tyr} is about 0.8×10^{-3} for the long wavelength phenolate CD bands of *cyclo*(-Phe-Tyr-) and *cyclo*(-Tyr-Tyr-), which is about the same as is observed for the unionized tyrosyl side chain of these same compounds at room temperature (see Table 3). These results are consistent with μ–μ coupling being the major mechanism generating the phenolate CD bands.

Studies with tyrosinate compounds provide only part of the information needed to interpret the effects of alkaline pH's upon the CD spectra of proteins. Increasing the pH of proteins above 7 may alter the conformation of aromatic side chains, of disulfide bridges, or of the peptide backbone due to ionizing tyrosyl or other groups, Ionization of cysteinyl side chains may perhaps produce a new CD band beginning at 270 nm and extending to shorter wavelengths, since its absorption band begins at this wavelength.[27] Irreversible changes, such as cleavage of disulfide bonds,[162] may also occur.

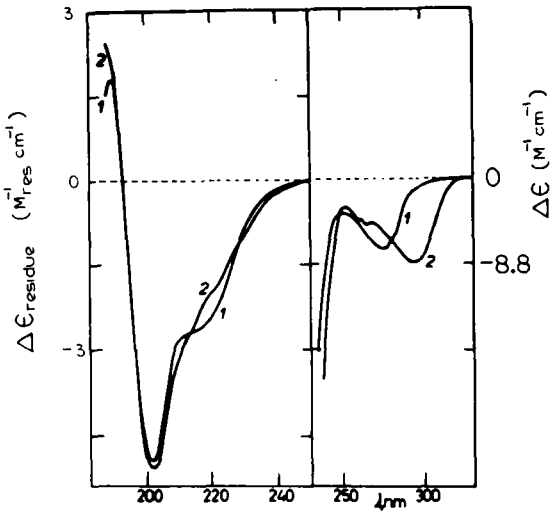

FIGURE 18. CD spectra of basic pancreatic trypsin inhibitor at room temperature: 2, 0.01 *M* NaOH; 1, 0.1 *M* Tris-HCl (pH 8). $\Delta\epsilon$ values were calculated for 58 amino acid residues per mole.[164] (From Fric et al.,[140] *Collect. Czech. Chem. Commun.*, 36, 2014, 1971. With permission.)

Not surprisingly, the alkaline pH's have been reported to produce a variety of CD spectra in proteins. The most interpretable case is where both the CD intensity and band shapes are unaffected by ionizing the tyrosyl side chain. Breslow[106] used this approach to identify the disulfide CD bands of neurophysin-II (see Figure 15). If the CD bands of a protein are altered at alkaline pH's, it is difficult, if not impossible, to prove that the orientation of aromatic side chains is unchanged. These CD spectra are useful mainly to identify conformational transitions and to determine whether aromatic side chains remain highly oriented at alkaline pH's.

CD spectra illustrate impressively the alkaline stability of basic bovine pancreatic trypsin inhibitor[140,163] (0 Trp, 4 Tyr, and 3 disulfides).[164] At pH 8, its negative CD band at 276 nm appears to have a vibronic shoulder (ca., 283 nm), indicating an appreciable tyrosyl CD contribution (see Figure 18). The absolute value of $\Delta\epsilon^{276}$ is 1.8 M_{Tyr}^{-1} cm^{-1} per tyrosyl, or less if there is an overlapping disulfide CD band. After treating the inhibitor with 0.01 *M* NaOH*, the negative CD band shifts to 294 nm, intensifies by 20%, and gains the shape expected for a tyrosinate CD band (see Figure 18). The relatively sharp onset of this band at 320 nm precludes any major disulfide contribution to the 294-nm band of the trypsin inhibitor. Apparently 3 to 4 tyrosyl side chains become ionized when the inhibitor is in 0.01 *M* NaOH.[165] Thus, the CD band at 294 nm has an average intensity of about -2.5 M_{Tyr}^{-1} cm^{-1} per tyrosinate ($|\Delta\epsilon|/\epsilon \approx 1 \times 10^{-3}$), which is about the largest value expected for a tyrosinate side chain (see Table 7). Evidently, dissolving this trypsin inhibitor in 0.01 *M* NaOH does not randomize its tyrosyl side chains. Their ionization, however, probably does cause some conformation change, because in the crystalline inhibitor, two tyrosyls are buried and seem to have their hydroxy groups hydrogen bonded to peptide carbonyls.[166]

In some proteins, the CD spectra are altered drastically at alkaline pH's. For example, arginine kinase of lobster (2 Trp, 11 Tyr, no disulfides)[167] has a negative CD plateau from 270 to 280 nm at pH 7.[168] Raising the pH to 10.3 or 11.9 almost completely abolishes the CD above 270 nm. Apparently, the aromatic side chains giving CD bands at pH 7 are relatively motile at pH 10.3 and above in arginine kinase.

Sometimes, the effects of pH change upon CD spectra may be most easily visualized by plotting the difference between the spectra measured at alkaline and neutral pH's.[169-171] Owing to the wide range of CD intensities possible at the two different pH's (states), these difference CD spectra may have numerous shapes. Four cases should be mentioned. The tyrosyl side chains may have strong CD in both their ionized and unionized states (e.g., human growth hormone),[170] only in their phenolate form (e.g., carboxypeptidase),[171] or only in their unionized state. The fourth possibility involves altered CD bands of other groups in addition to the tyrosyl-tyrosinate CD changes with pH.

***O*-Acylated tyrosyl side chain** – Numerous CD measurements have been reported for proteins acetylated with *N*-acetylimidazole or acetic anhydride.[112,115,133,139,155,172-179] This modification is potentially one of the most useful for identifying tyrosyl CD bands in proteins. *O*-Acetylation converts the near-UV tyrosyl absorption bands into phenylalanyl-like

*Based upon the discussion in the text of Reference 140, the concentration seems to be 0.01 *M* even though their figure legend indicates 0.1 *M* NaOH.

bands.[158,180] When *O*-acetylation is complete, the tyrosyl vibronic absorption bands above 275 nm disappear completely.[115] Thus, one would anticipate that the near-UV CD spectra of *O*-acetylated tyrosyls may resemble those described for phenylalanyl. Apparently, no CD studies have been made on tyrosyl model compounds shown to be fully *O*-acetylated, although some preliminary spectra have been reported.[172,179]

Above 275 nm, the CD changes caused by acetylating a protein may perhaps reflect the loss of tyrosyl CD arising from the side chain that was acetylated. Unfortunately, other possibilities exist. If the acetylated tyrosyl is located near non-acetylated tyrosyls, tryptophanyls, or disulfides, their CD may be altered indirectly. Far-UV transitions of the tyrosyl side chain are also affected by *O*-acetylation, in part because the ester moiety introduces several additional transitions.[44,181] Thus, coupling of these far-UV transitions with the near-UV bands of neighboring side chains may be changed in the acetylated protein. Among the chemical modifications that alter the near-UV tyrosyl band, however, acetylation probably has the least effect upon the far-UV transitions.

Three other possible difficulties with the acetylation technique deserve mention here. First, since the acetoxy group is much bulkier than the hydroxy group, their interactions with other moieties may differ sufficiently to produce somewhat different protein conformations.

Secondly, the acetylating agent (*N*-acetylimidazole or acetic anhydride) may also acetylate other groups in addition to the tyrosyl side chain.[112,180] For this reason, it is essential to compare the CD spectrum of the native protein with the spectrum obtained after selectively deacetylating the tyrosyl side chains with hydroxylamine at neutral pH.[173,175,177] To the extent these CD spectra differ, acetylation of nontyrosyl groups may also be altering the near-UV CD bands. With α-lactalbumin, major irreversible CD alterations occurred when higher ratios of *N*-acetylimidazole to protein were used.[173] In the case of lysozyme, tryptophanyl CD was altered by the unwanted acetylation of amino groups.[175]

Thirdly, estimating the number of tyrosyls acetylated may be subject to appreciable error in some cases. The spectrophotometric assay is uncertain, because tyrosyl side chains of proteins may differ in absorption intensity from that observed for model compounds in water.[115,158] Owing to band sharpening, wavelength shifting, and hydrogen bonding of the phenolic hydroxy group, ϵ values in proteins may be as much as 30% higher than the value observed in water.[74,182] Complete acetylation of tyrosyls, however, is easily determined from the absorption spectrum, if a protein lacks tryptophanyls or a prosthetic group with near-UV absorption. In these cases, incomplete acetylation will be evident from tyrosyl fine structure above 275 nm, especially in absorption spectra at 77°K.[115]

By carefully analyzing the CD and absorption spectra of acetylated proteins, it may be possible to identify CD contributions of individual tyrosyls. Then, the method of Burstein and Patchornik[183] may be used to determine which tyrosyls have been acetylated.

Oxidized tryptophanyl side chain – Attempts have been made to analyze the tryptophanyl CD bands of some proteins[145,172,178,184-189] by oxidizing with either *N*-bromosuccinimide[190] or iodine.[191] Often, these agents convert the tryptophanyl residue to a β-3-oxindolylalanyl residue, which has absorption bands at 250 and 280 nm.[192,193] The oxindolylalanyl band at 280 nm is broad, has moderate intensity,* and lacks the sharp vibronic structure of the ^1L_b tryptophanyl band. The 250-nm oxindolylalanyl band is relatively intense (ϵ^{max}, 7,000 to 8,000 M^{-1} cm^{-1}). Both these bands have appreciable CD intensities in *N*-acetyl-β-3-oxindolyl-L-alanine ethyl ester.[193] Apparently, the oxindolyl side chains of proteins should be capable of having appreciable near-UV CD intensity, when favorable interactions occur with other moieties.

Other potential difficulties include the variety of products that may be formed from the original tryptophanyl side chains[189,190,193,194] and side reactions which may occur with other amino acid residues.[195,196] Some spectra obtained from oxidizing tryptophan with *N*-bromosuccinimide differ appreciably from spectra of oxindolylalanine, especially in the region above 300 nm.[172,191,197] In addition, some tautomeric forms of oxindolylalanine may be preferentially stabilized by reactions with neighboring side chains of a protein.[189,193,194] Depending upon which tautomeric form is produced, the modified

*The ϵ^{280} varies from about 1,000 to 1,500 M^{-1} cm^{-1} depending upon the overlap from the short wavelength band.

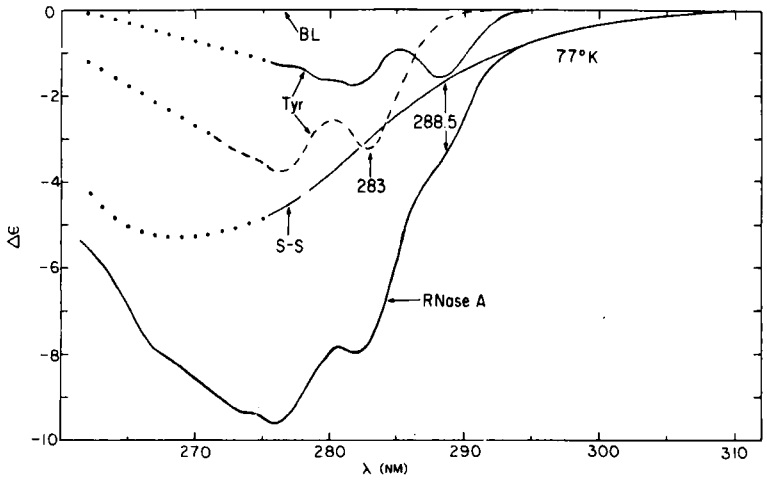

FIGURE 19. Analysis of ribonuclease-A CD spectrum at 77°K. S-S designates disulfide CD. (Reprinted from Horwitz et al.,[49] *J. Am. Chem. Soc.*, 92, 2123, 1970, © 1970 by the American Chemical Society. Reprinted by permission of the copyright owner.)

tryptophanyl side chain may exhibit one of several different types of CD spectra.[193] In all cases, the modified side chain may have CD bands that overlap the naturally occurring near-UV CD bands in proteins.[172,193] Thus, the CD spectrum of a protein oxidized with *N*-bromosuccinimide or iodine may sometimes be even more complex than the spectrum of the native protein, e.g., soybean trypsin inhibitor,[172] lysozyme,[188] and dihydrofolate reductase.[185]

ANALYSIS OF CD SPECTRA OF SELECTED PROTEINS

This section illustrates the identification of near-UV CD bands in proteins by using wavelength profile, chemical modification, and crystal structure information, if available. In the case of proteins lacking tryptophanyl side chains, a moderately detailed understanding may be possible. As the number of tryptophanyl side chains increases, interpretation of CD spectra becomes more difficult.

Ribonuclease

The near-UV CD spectra of bovine pancreatic ribonuclease-A and -S have been studied in some detail.[42,49,137,174,179,198-212] Earlier work has been reviewed.[1,2,5,6] Analysis of these CD spectra is facilitated by the wealth of information about ribonuclease,[213] which has 6 tyrosyls, 3 phenylalanyls, 4 cystinyls and lacks tryptophanyl.

Wavelength profile analysis – Low-temperature spectra have permitted a detailed analysis of the moieties contributing to the CD and absorption of ribonuclease-A.[49] At 77°K, its CD spectrum has well-resolved fine structure at 283 and 276 nm and shoulders at 288.5, 267.5, 261, and 255 nm (see Figure 19). The fine structure at 267.5, 261, and 255 nm arises from phenylalanyl side chains. These fine-structure CD bands are superimposed upon a much more intense background arising from the tyrosyl and cystinyl side chains. The 288.5-nm CD shoulder indicates the position of the 0–0 transition of a single buried tyrosyl. The characteristic tyrosyl fine structure at 283 (0–0) and 276 nm (0 + 800 cm^{-1}) results from 3 tyrosyl side chains having their hydroxy groups exposed to the solvent. Two buried tyrosyl side chains, whose 0–0 transition is prominent in the absorption spectrum at 286 nm (see Figure 20), do not have measurable CD (see Figure 19). The broad ribonuclease-A CD band beginning about 310 nm and extending toward shorter wavelengths must be the leading edge of a disulfide CD band, because tyrosyl CD does not extend above 295 nm at 77°K. Based upon a simultaneous fitting of the ribonuclease-A CD and absorption spectra using data from tyrosyl model compounds, Horwitz et al.[49] suggested that the disulfide CD of ribonuclease-A peaks near 270 nm (see Figure 19).

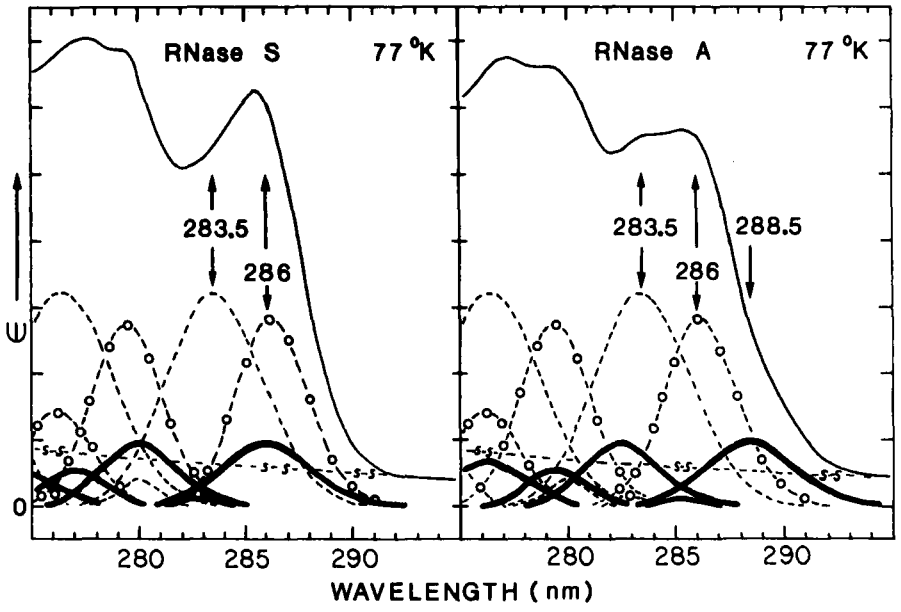

FIGURE 20. Comparison of absorption spectra of ribonuclease-A and -S at 77°K. -S-S-, disulfide. In ribonuclease-A, three tyrosyl sites are identified in terms of the positions of the 0–0 bands: –, 288.5 nm; 0-0, 286 nm; - - -, 283.5 nm. The absorption of each tyrosyl site is represented by bands corresponding to the vibronic transitions of tyrosyl. The ribonuclease-S spectrum was synthesized from the bands observed in ribonuclease-A except that the contribution of a single tyrosyl (0–0 at 288.5 nm in A) was shifted 2.5 nm to shorter wavelength. (From Horwitz and Strickland, *J. Biol. Chem.*, 246, 3750, 1971. With permission.)

Subsequent studies[106] confirmed that the CD intensity and shape proposed for the disulfide band of ribonuclease-A are comparable to those observed in some model systems (see Table 5).

The CD of ribonuclease-A, at room temperature, is similar to that at 77°K except for having less sharp fine structure.[49] Even at room temperature, the tyrosyl CD shoulder at 288.5 nm may permit measuring differences in the environment of a single tyrosyl (probably Tyr-25).[137] A glycosylated bovine pancreatic ribonuclease may perhaps have an even more prominent CD shoulder at 288.5 nm than does ribonuclease-A (compare Figure 1 in Reference 212 and Figure 2 in Reference 210). The 288.5-nm band, however, is absent in the CD spectra of ribonuclease-A that has been treated with subtilisin to cleave a single peptide bond (ribonuclease-S in Figure 21).[137] At least in part, the disappearance of this CD band results from its being shifted to a shorter wavelength in ribonuclease-S. By comparing absorption spectra recorded at 77°K, one can see that the 288.5-nm shoulder in the ribonuclease-A spectrum has shifted about 2.5 nm toward shorter wavelengths in the ribonuclease-S spectrum (see Figure 20). Not surprisingly, this shift cannot be observed in absorption spectra recorded at room temperature where the broader bands obscure small spectral shifts.[214] Thus, when the CD of a single tyrosyl is relatively intense, its wavelength position may sometimes be seen more clearly in the CD spectrum than in the absorption spectrum.

Interactions giving tyrosyl CD – Recently, Strickland[42] has examined theoretically the tyrosyl CD bands of ribonuclease-S by using the atomic coordinates of the crystalline enzyme. For comparison of these theoretical results with the experimental spectrum, one should note that the hydroxy groups of Tyr-73, -76, and -115 are free to interact with the solvent (0–0 transition expected near 283 nm). In contrast, the hydroxy groups of Tyr-25, -92, and -97 are hydrogen bonded to acceptor groups belonging to the protein[213] which should red shift their 0–0 transition.[74] The tyrosyl CD of ribonuclease-S was assumed to result principally from coupling between the near-UV tyrosyl band and the $\pi-\pi^*$ transitions located on other moieties. These calculations revealed that a variety of interactions contribute some of the tyrosyl CD (see Table 8).

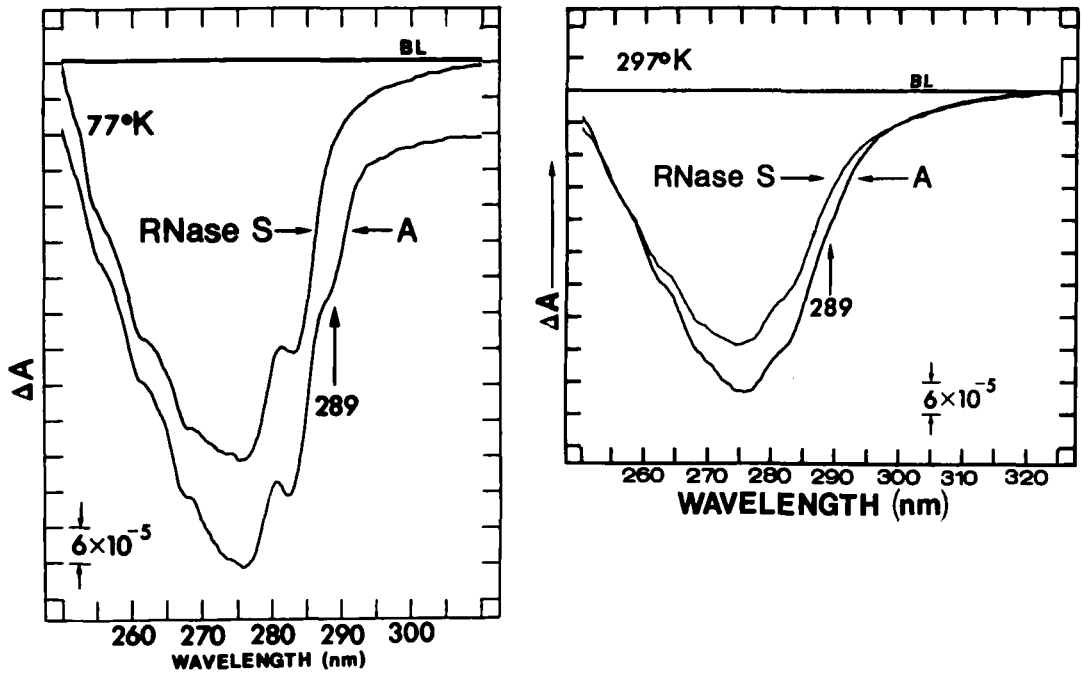

FIGURE 21. Comparison of CD spectra of ribonuclease-S and -A at 77°K (left) and 297°K (right). (From Horwitz and Strickland, *J. Biol. Chem.*, 246, 3751, 1971. With permission.)

The contributions from μ–μ coupling with peptide transitions are of special interest, because the tyrosyl side chains of any protein are likely to be located near several peptide bonds. In the case of ribonuclease-S, the absolute value of $\Delta\epsilon^{max}$ from all interactions with peptide bonds ranges from 0 to 1 M^{-1} cm^{-1} for individual tyrosyl side chains. For each of the six tyrosyls, there are numerous interactions with different peptide bonds (see Figure 22). Some interactions give positive CD; others give negative CD. Owing to this cancellation, the net CD for each tyrosyl is not much larger than the CD resulting from some of the single interactions. For example, the interaction of Tyr-76 with peptide bond 60 gives a rotatory strength of 1.7 x 10^{-40} c.g.s., which is approximately equivalent to a $\Delta\epsilon^{max}$ of 0.85 M^{-1} cm^{-1} (see Figure 22). The sum for the interactions between Tyr-76 and the remaining peptide bonds adds only an additional 0.05 M^{-1} cm^{-1} to the

TABLE 8

Near-UV Tyrosyl CD Bands of Ribonuclease-S Resulting from μ-μ Coupling[a]

All Interactions with	$\Delta\epsilon^{max}$ for Tyrosyl Side Chain #[b]					
	73	115	76	25	92	97
Other Tyr side chains	−1.9	−1.8	0.2	0.2	0.0	0.4
Phe side chains	0.0	0.0	0.0	−0.3	0.0	−0.2
Peptide bonds	−0.6	−0.7	0.8	0.3	−0.2	−1.2
His side chains	0.0	0.0	0.0	−0.5	0.0	0.0
Total $\Delta\epsilon^{max}$	−2.5	−2.5	1.0	−0.3	−0.2	−1.0

[a]Modified from Reference 42.
[b]R_s converted to $\Delta\epsilon^{max}$ using $\Delta\epsilon^{max} \approx 0.5 \times R_s \times 10^{40}$ (for 297°K)[74].

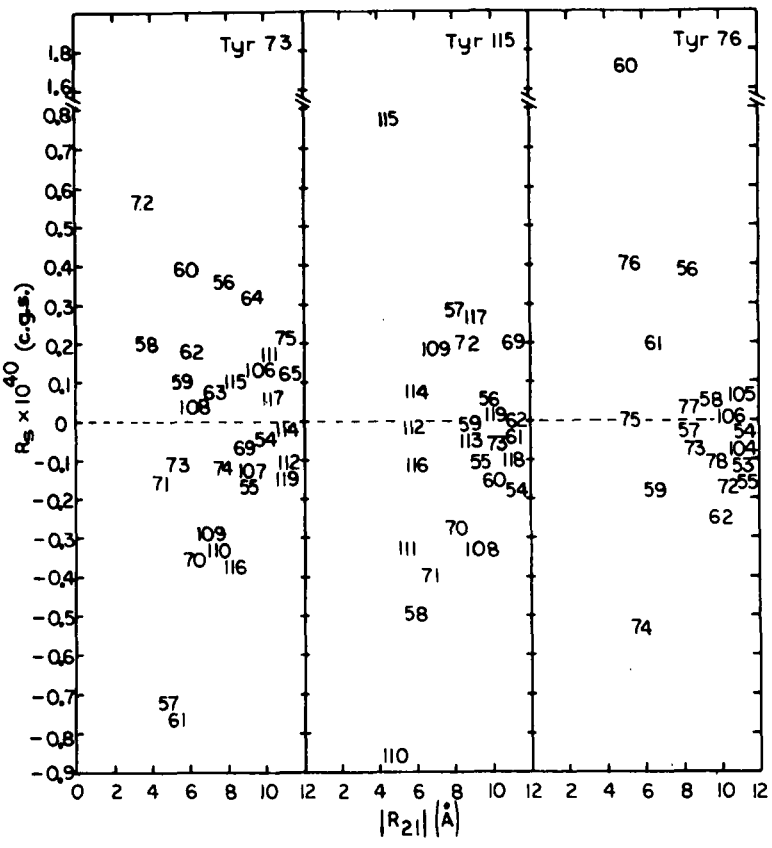

FIGURE 22. Effect of distance upon the CD strength of Tyr-73, -115, and -76 for coupling with $\pi-\pi^*$ peptide transitions (190 nm) of ribonuclease-S. $|R_{21}|$ is the distance between the center of a tyrosyl ring and the center of a peptide bond. Each number designates which peptide bond is involved. The rotatory strengths (R_s) can be converted approximately to $\Delta\epsilon^{max}$ by the equation $\Delta\epsilon^{max} = 0.5 \times R_s \times 10^{40}$. (Reprinted from Strickland, *Biochemistry*, 11, 3469, 1972, © 1972 by the American Chemical Society. Reprinted by permission of the copyright owner.)

$\Delta\epsilon^{max}$.

The data in Figure 22 also illustrate the distance dependence of CD. Although the CD intensity tends to get larger for peptide bonds located closer to the tyrosyl side chain, there is also a geometry factor. Thus, if the geometry is unfavorable, the interaction may be weak even for groups in contact with the tyrosyl ring. In most cases, the interactions contribute insignificant rotatory strength when the distance is more than 8 to 10Å between the center of the peptide bond and the center of the tyrosyl ring.

In the case of ribonuclease-S, much of the near-UV tyrosyl CD results from interactions between aromatic side chains. The group of interactions between Tyr-73 and Tyr-115 contribute a large CD intensity ($\Delta\epsilon^{275} \approx -3.7\ M^{-1}$ cm^{-1}, see Table 9). Coupling between these neighboring tyrosyls, located on the surface, produces about 40% of the $\Delta\epsilon^{275}$ observed for ribonuclease-S. The total interaction between these tyrosyls includes coupling of the near-UV band of each side chain with the far-UV aromatic bands of the other side chain. In addition to the $\Delta\epsilon$ values shown in Table 9, there is a small exciton CD band due to coupling between the near-UV CD bands of these two tyrosyls.[42] Tyr-25 is the only other tyrosyl located near aromatic side chains. The near-UV CD resulting from these interactions, however, is small (see Table 9).

By assigning wavelength positions and band shapes for each tyrosyl CD band, a calculated spectrum has been obtained for the total tyrosyl CD of ribonuclease-S (Σ Tyr in Figure 23). This

TABLE 9

Near-UV Tyrosyl CD of Ribonuclease-S Resulting from Coupling with Transitions in Other Tyrosyl, Phenylalanyl, and Histidyl Side Chains Whose Ring Centers Are Closer than 11 Å[a]

Near-UV Band of Tyr	Coupling with Far-UV Bands on Side Chain	R_{21},[b] Å	$\Delta\epsilon,^{max}$ M^{-1} cm^{-1}
73	Tyr-115	6	-1.9
	Tyr-76	10	0.0
115	Tyr-73	6	-1.8
76	Tyr-73	10	0.1
	His-105	9	0.0
25	Phe-46	6	-0.1
	His-48	6	-0.6
97	Phe-46	10	-0.3

[a]Modified from Reference 42.
[b]R_{21}, distance between ring centers.

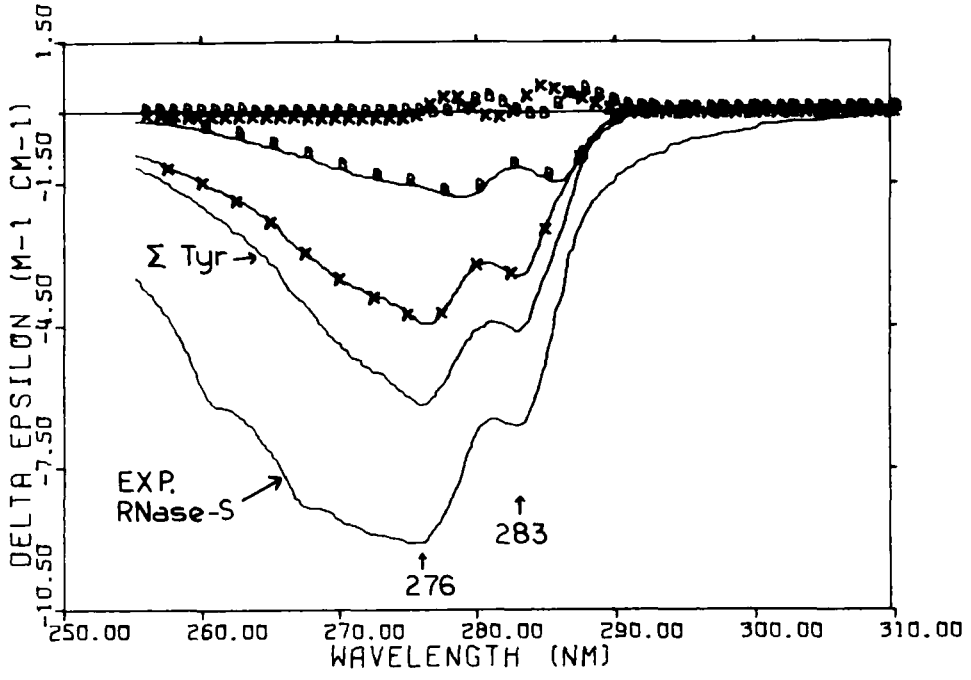

FIGURE 23. Comparison between the tyrosyl CD bands calculated for ribonuclease-S and the spectrum actually observed for ribonuclease-S at 77°K (EXP. RNase-S). Σ Tyr, total for individual tyrosyl CD bands and their exciton components; X-X, CD spectrum of Tyr-73, -115, and -76; B-B, CD spectrum of Tyr-25, -92, and -97; X X X, exciton CD component for Tyr-73 -115 interaction; B B B, exciton CD component for sum of Tyr 25-97 and Tyr 92-97 interactions. (Reprinted from Strickland, *Biochemistry*, 11, 3472, 1972, © 1972 by the American Chemical Society. Reprinted by permission of the copyright owner.)

spectrum reliably reproduces the shape of the tyrosyl vibronic structure observed experimentally but is less intense than the experimental trace, especially below 270 nm. The disulfide CD band of ribonuclease-S probably accounts for most of the intensity difference between these two curves.[42]

The calculated CD spectrum of ribonuclease-S

also provides a qualitative basis for considering the tyrosyl CD bands of ribonuclease-A, since their structures are similar in most regions, except where the single peptide bond is cleaved by subtilisin.[213] The calculations for ribonuclease-S suggest that the CD intensity of Tyr-25 may be sensitive to a local conformation change that may result from subtilisin treatment of ribonuclease-A.[42] Among the other tyrosyls of ribonuclease-A, the interactions between Tyr-73 and Tyr-115 are probably a major source of near-UV CD.

The CD spectrum of rat ribonuclease[215] gives experimental support to the large CD predicted from the interactions between Tyr-73 and Tyr-115 in the bovine enzymes. The conformation of the peptide backbone seems to be similar in the two species, even though there are 41 amino acid substitutions.[216,217] The rat enzyme has only 4 tyrosyls (25, 92, 97, and 115), Tyr-73 being replaced by His and Tyr-76 by Ser. The effects of losing Tyr-73 and Tyr-76 can be estimated from the data in Tables 8 and 9. First, there is a direct loss of the CD from these tyrosyls (+ 1 M^{-1} cm^{-1} for 76 and -2.5 M^{-1} cm^{-1} for 73). In addition, the CD of Tyr-115 is altered because its ^1L_b transition can no longer interact with the transitions belonging to the side chain of Tyr-73 ($\Delta\epsilon \approx$ -1.8 M^{-1} cm^{-1}). Thus, the total loss of CD is about -3.3 M^{-1} cm^{-1}. The rat enzyme, however, may have some additional CD resulting from the new interactions between Tyr-115 and His-73. The latter interactions may tend to produce less CD, because the imidazolyl transitions are weaker and located at much shorter wavelengths than are the far-UV transitions of the tyrosyl side chains. In agreement with the approximate calculation presented above, the experimentally reported $\Delta\epsilon^{275}$ value for rat ribonuclease shows a loss of about -3.5 M^{-1} cm^{-1} relative to the value for the bovine enzyme.[215]

Effects of modifying side chains – In earlier years, the effects of alkaline pH's upon the ribonuclease-A CD spectrum led to much controversy about the origin of the near-UV CD bands.[179,199,200] Unfortunately, these earlier spectra were recorded on high-noise CD instruments which did not resolve subtle features in the ribonuclease-A CD spectra. Even at room temperature, the CD spectrum at pH 11.5 has sufficient detail to identify the aromatic CD bands (see Figure 24).[76] Raising the pH from 7 to 11.5 removes the CD band at 275 nm and produces a

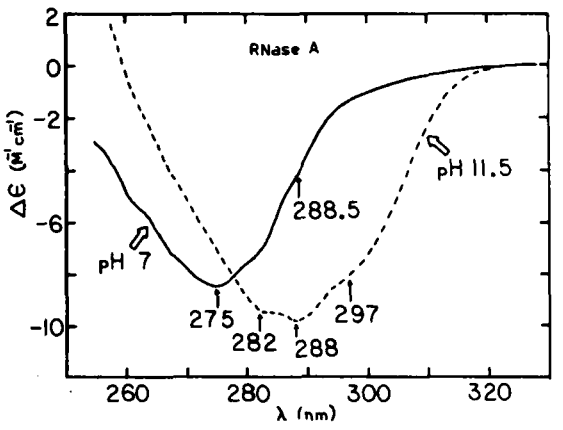

FIGURE 24. Comparison of ribonuclease-A CD spectrum at pH 7 (1 m*M* sodium phosphate buffer) and at pH 11.5 after adding NaOH to same solution. 297°K. (From Strickland and Billups.[76])

shoulder extending from 302 to 292 nm. These CD alterations mainly reflect ionization of the three tyrosyls with exposed hydroxy groups (Tyr-73, -76, -115). Interestingly, the intensity of the tyrosinate CD band (change in $\Delta\epsilon^{300} \approx -6$ M^{-1} cm^{-1}) is greater than that of the unionized exposed tyrosyl side chains ($\Delta\epsilon^{275} \approx -4$ M^{-1} cm^{-1}) (from resolved spectrum in Figure 19). This change probably includes both conformational and electronic effects. Ionizing the neighboring hydroxy groups on Tyr-73 and -115 may alter the orientation of their side chains. Also, the CD generated by μ–μ coupling will tend to increase due to the 65% greater absorption intensity of the tyrosinate side chain. From a qualitative viewpoint, the high tyrosinate CD intensity (ratio of change in $\Delta\epsilon^{300}$ to change in $\epsilon^{300} \approx 1 \times 10^{-3}$) suggests that the side chains of Tyr-73 and Tyr-115 remain in proximity and fairly rigid even in the ionized state.

In addition to the tyrosinate CD bands, the ribonuclease-A CD spectrum at pH 11.5 has tyrosyl bands at 288 nm (0–0) and 282 nm (0 + 800 cm^{-1}). Even at pH 11.5 there is still no measurable tyrosyl CD band at 286 nm, which would correspond to the 0–0 absorption band of two buried tyrosyls (92 and 97).

Chemical modifications of tyrosyl and cystinyl side chains have not yielded much information about the near-UV CD bands of ribonuclease-A. Acetylating tyrosyl side chains has given conflicting results. Simons[174] reported a 50% loss of CD intensity at 275 nm, whereas Pflumm and

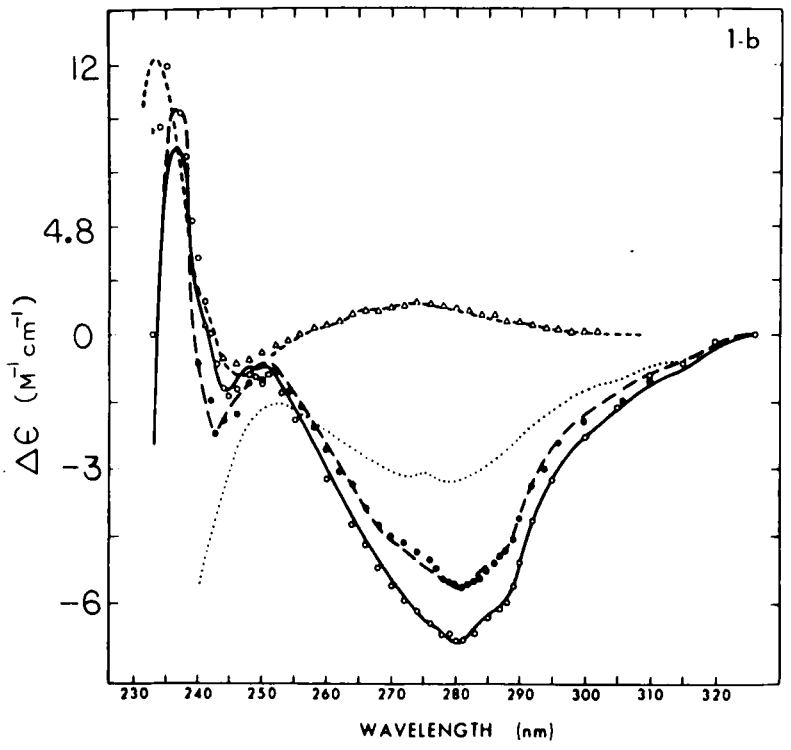

FIGURE 25. CD spectra of luteinizing hormone (–), α subunit (Δ), and reassociated molecule (○) in 0.1 *M* phosphate buffer (pH 6.0). Other spectra were recorded in 0.1 *M* Tris-phosphate containing 5% dioxane (pH 7.5): luteinizing hormone (– – –), α subunit (- - - -), β subunit (· · · ·), and reassociated molecule (●). (Reprinted from Bewley et al.,[116] *Biochemistry*, 11, 934, 1972, © 1972 by the American Chemical Society. Reprinted by permission of the copyright owner.)

Beychok[179] found only a slight change when three tyrosyls were claimed to be acetylated. Nitration studies[179,202] have not given sufficient attention to the 280-nm CD band of the nitrotyrosyl side chain.[140] Cleaving the four disulfide bonds of ribonuclease-A largely abolishes the near-UV CD.[201,203,207] Apparently, some of the disulfide bonds are necessary to maintain the native conformation of ribonuclease-A.

Luteinizing Hormone (Interstitial Cell-Stimulating Hormone)

Bewley, Sairam, and Li[116] have interpreted the CD spectrum of this glycoprotein hormone obtained from sheep pituitaries. Luteinizing hormone, which lacks tryptophanyl, has two subunits held together by noncovalent interactions. The α subunit has 5 tyrosyls, 5 phenylalanyls, and 5 disulfide bonds among its 96 residues, whereas the β subunit has 2 tyrosyls, 3 phenylalanyls, and 6 disulfide bonds among its 120 residues.[164] Evidently, the CD of luteinizing hormone above 255 nm results from both disulfide bonds and tyrosyl side chains (see Figure 25).[116] The long wavelength edge of the disulfide CD can be seen beginning near 325 nm. Below 300 nm the tyrosyl side chains of the native luteinizing hormone may have appreciable CD intensity, because the characteristic tyrosyl vibronic structure occurs prominently at 281 and 287-288 nm (see Figure 25). Apparently, the 288-nm shoulder indicates the position of the 0–0 tyrosyl CD band. This relatively long wavelength for the 0–0 band implies that it arises from "buried" tyrosyl side chain(s).

Ovomucoid

This glycoprotein has a molecular weight of 27,300 and contains 6 tyrosyls, 8 disulfides, 5 phenylalanyls, and no tryptophanyls.[218]

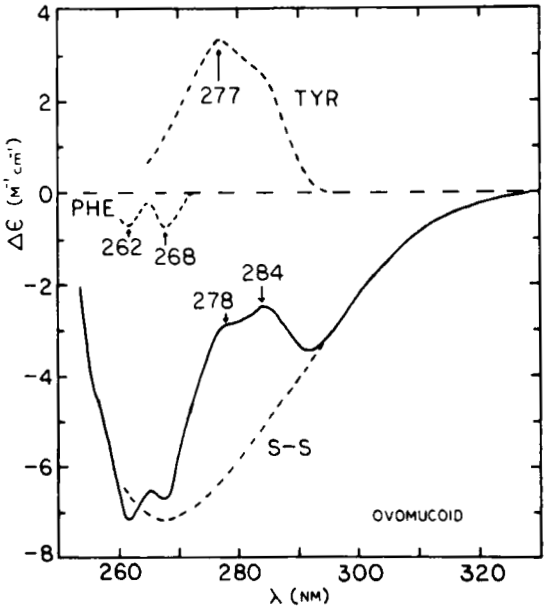

FIGURE 26. CD spectrum of chicken ovomucoid (pH 7.5) at 297°K (–). - - - -, indicates contributions proposed for Tyr, Phe, and disulfide bonds. (From Kay et al.,[115] *J. Biol. Chem.*, in press. With permission of the copyright owner.)

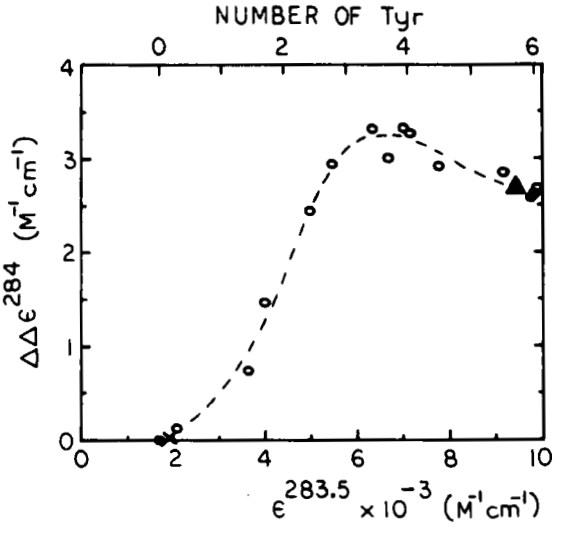

FIGURE 27. Intensity of tyrosyl CD band ($\Delta\Delta\epsilon^{284}$) plotted against the number of tyrosyls remaining unacetylated in ovomucoid at 297°K. (From Kay et al.,[115] *J. Biol. Chem.*, in press. With permission of the copyright owner.)

Gorbunoff[112] examined its CD spectrum by acetylating various numbers of tyrosyls with *N*-acetylimidazole. Above 260 nm the CD spectrum was altered only slightly even when an average of 5.9 of the 6 tyrosyls were reported to be acetylated. Thus, she suggested that the near-UV CD of ovomucoid originated mainly from disulfide bonds and phenylalanyls. Her suggestion, however, seems incompatible with the wavelength profile of the ovomucoid CD spectrum.

For this reason, Kay et al.[115] reinvestigated the origin of the ovomucoid CD bands by using wavelength profile analysis and by acetylating the tyrosyl side chains. Their analysis indicates that tyrosyls, disulfides, and phenylalanyls contribute to the CD of native ovomucoid (see Figure 26). The phenylalanyls give a pair of weak, sharp bands at 268 and 262 nm. The negative CD band beginning about 330 nm and extending to its maximum near 268 nm arises from disulfide bonds. Superimposed on this broad band is a narrow positive CD contribution arising from the tyrosyls. The characteristic tyrosyl CD fine structure produces the bands at 284 and 278 nm. The apparent negative band at 292 nm is actually a pseudoband induced by the overlap of the narrow tyrosyl CD structure upon the broad disulfide CD band.

Kay et al.[115] found that the positive CD bands at 284 and 278 nm were abolished by fully acetylating the 6 tyrosyls of ovomucoid. This acetylation caused only slight changes in the negative disulfide CD band above 275 nm. Deacetylating the tyrosyls with hydroxylamine regenerated the positive CD bands at 284 and 278 nm, suggesting that these bands may arise from tyrosyls.

The difference between the findings of Kay et al.[115] and Gorbunoff[112] may be related to uncertainties in estimating the number of tyrosyls acetylated in ovomucoid. In agreement with Donovan,[219] Kay et al. found that the tyrosyls in ovomucoid have about 20% more intense molar absorptivity than is the case for tyrosyls dissolved in water. This hyperchromism may have caused Gorbunoff[112] to overestimate the number of tyrosyls acetylated. Kay et al.[115] reported that the last two tyrosyls of ovomucoid could only be acetylated with *N*-acetylimadazole when a denaturing agent was also present. Since Gorbunoff did not use a denaturing agent during acetylation, her most acetylated preparation probably retained two tyrosyls.

Kay et al. studied the tyrosyl CD fine structure height as the number of tyrosyls in ovomucoid was diminished by *O*-acetylation (see Figure 27). Ace-

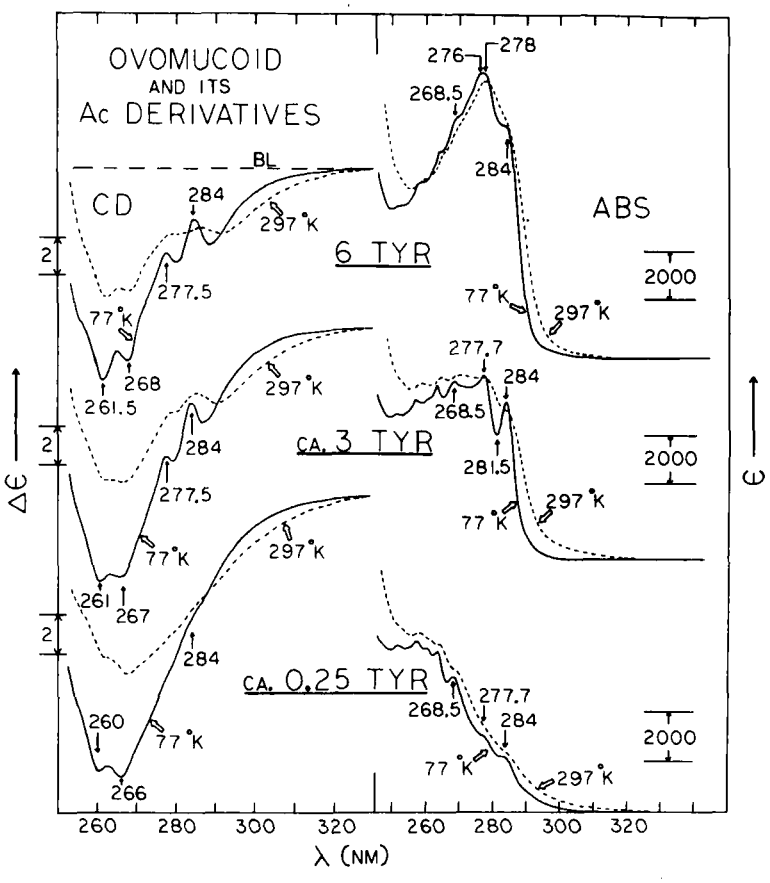

FIGURE 28. 297 and 77°K CD (left) and absorption (right) spectra of ovomucoid (top), partially acetylated ovomucoid (middle), and almost completely acetylated ovomucoid (bottom) at pH 7. (From Kay et al.,[115] *J. Biol. Chem.*, in press. With permission of the copyright owner.)

tylating the first two tyrosyls intensifies the tyrosyl CD fine structure at 284 nm by 25%. Perhaps these two tyrosyls have a net negative CD (-0.3 M^{-1} cm^{-1} per tyrosyl). Alternatively, this partial acetylation may alter the orientation of the remaining tyrosyls or may affect μ–μ coupling with them. Acetylating the last three tyrosyls abolishes the tyrosyl CD bands. The average CD intensity of these three tyrosyls is appreciable (1.1 M^{-1} cm^{-1} per tyrosyl at 297°K).

A conclusive identification of tyrosyl fine structure was made from low-temperature CD and absorption spectra of ovomucoid and its acetylated derivatives dissolved in a water-glycerol buffer. From an analysis of these spectra (see Figure 28), Kay et al.[115] drew the following conclusions. About 2 tyrosyls have their 0–0 absorption band at about 282 nm and about 4 tyrosyls have their 0–0 absorption band at 284 nm. The 2 tyrosyls with 0–0 bands at 282 nm are most easily acetylated and have only weak CD. The 3 tyrosyls that contribute most of the tyrosyl CD have their 0–0 CD and absorption bands at 284 nm. Their 0 + 800 cm^{-1} transition occurs prominently at about 277.5 nm. Since these tyrosyl bands are much sharper than those observed with L-tyrosine dissolved in water-glycerol at 77°K,[49] the tyrosyl side chains giving most of the CD in ovomucoid must be appreciably shielded from the water-glycerol solvent.

Two other aspects of these low-temperature experiments deserve mention.[115] First, the glycerol required for 77°K spectra causes some decrease in the intensity of the tyrosyl CD bands of ovomucoid at room temperature. Second, cooling ovomucoid gradually enhances the rotatory strength of the disulfide CD band, suggesting that some conformational change may occur.

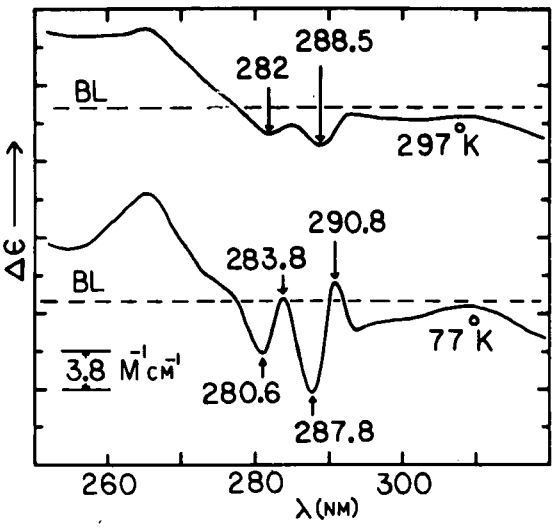

FIGURE 29. CD spectra of horse heart ferricytochrome c dissolved in water-glycerol (1:1, v:v) containing 100 m*M* sodium phosphate (pH 7) at 297 and 77°K. Control experiments showed that adding 50% glycerol to the aqueous buffer does not alter the near-UV CD spectrum of ferricytochrome c at 297°K. BL indicates approximate position of base line. Absorption spectra of these samples are shown in Figure 6 of Reference 94. (From Strickland, Horwitz, and Billups.[226])

Although the conformation of ovomucoid at 77°K appears different from that at 297°K in an entirely aqueous system, their CD spectra have similar wavelength profiles. Apparently, the same moieties contribute to both CD spectra, even though their relative intensities differ somewhat.

Insulin

The near-UV CD studies on insulin[86,138,139,198,220] have been ably reviewed by Sears and Beychok.[2] Apparently, its major near-UV CD band (275 nm) arises partly from tyrosyl side chains and partly from disulfide bonds.

Cytochrome c

The protein isolated from horse heart contains one tryptophanyl, four tyrosyls, four phenylalanyls, no disulfides, and one covalently bound heme. The single tryptophanyl and three of the tyrosyls are invariant in all species studied.[157] Analysis of cytochrome c spectra is complicated by the broad, relatively intense heme CD bands occuring in the near-UV.[117] In 1967 Urry[117] suggested that fine structure could be used to assess the CD of aromatic amino acid side chains belonging to heme proteins, because the heme group lacks fine structure in the near-UV (see Other Groups Which May Have Near-UV CD). At room temperature, the ferricytochrome c CD spectra of many species have a pair of negative vibronic bands at 282 and 288 to 289 nm.[117,221-225] Initially, Myer[224] and also Urry[221] suggested that these vibronic CD bands probably arise from the tryptophan residue.

Recently, Myer[184,196] has reexamined the origin of the 282 and 288-nm CD bands by oxidizing horse heart ferricytochrome c with *N*-bromosuccinimide. The modified amino acid residues were determined for a range of *N*-bromosuccinimide to protein ratios. The first set of modifications seems to be largely completed at about 3.5 equivalents of *N*-bromosuccinimide per mole of cytochrome c. At this point, Trp-59, Met-65, and one tyrosine (74?) have been oxidized completely. These modifications do not abolish the negative CD bands at 282 and 288 nm, although the trough between them seems to become shallower (see Figure 2 in Reference 184). The persistence of these negative vibronic bands strongly suggests that they do not arise from tryptophanyl.[184] Even though the oxidized tryptophanyl side chain has not been thoroughly characterized,* it seems highly unlikely that the oxidized side chain could have sharp vibronic bands at exactly the same wavelength as tryptophanyl. Apparently, the 282- and 288-nm CD bands of ferricytochrome c arise from tyrosyl side chains.

The origin of these bands has been examined further by Strickland, Horwitz, and Billups,[226] using the CD spectrum of ferricytochrome c at 77°K (see Figure 29). Cooling greatly sharpens the negative vibronic bands (287.8 and 280.6 nm) and also reveals weaker positive bands at 290.8 and 283.8 nm. No obvious CD band occurs near 293 nm, which is the wavelength of the 0–0 ^1L_a tryptophanyl absorption band.[94] The broad positive CD band at 265 nm arises from the heme moiety.[117] A weak broad positive CD band may occur at 275 nm. Careful comparison of the CD

*Based upon the absorption decrease at 280 nm (see Figure 1 in Reference 196), Myer[184,196] suggested that the product is oxindolylalanyl. This interpretation, however, fails to explain the absence in oxidized cytochrome c of the intense 250-nm absorption band characteristic of the oxindolyl side chain.[192,193]

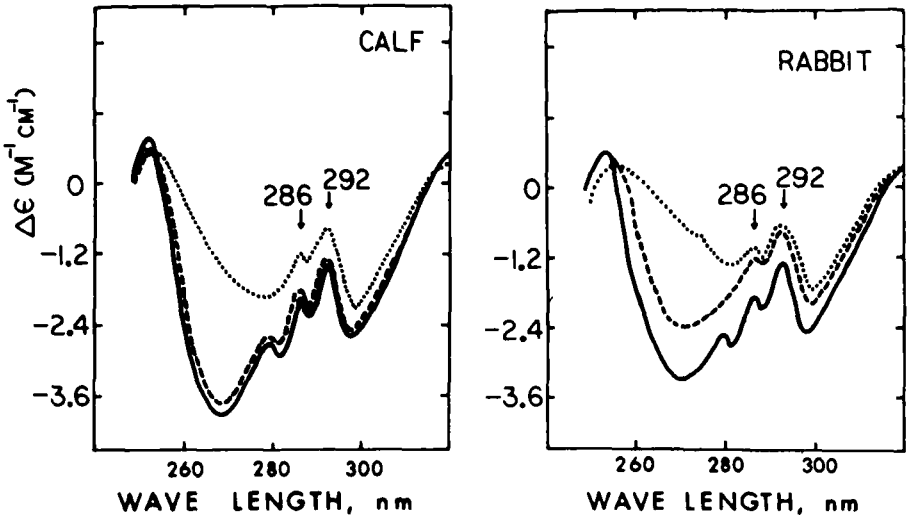

FIGURE 30. CD spectra of cytochrome b_5 (pH 8.1,298°K). Left: –, calf liver cytochrome b_5 (99 amino acid residues); - - -, its tryptic heme peptide (88 residues); · · ·, acetylated cytochrome b_5. Right: –, rabbit cytochrome b_5; · · · ·, its acetylated derivative; - - -, acetylated derivative after incubation with hydroxylamine to deacetylate tyrosyls. (From Huntley and Strittmatter, *J. Biol. Chem.*, 247, 4644, 1972. With permission.)

and absorption using the same monochromator and time constant confirmed that the 0–0 ^1L_b tryptophanyl absorption band[94] lies between the 290.8 and the 287.8-nm CD bands. Apparently, the 0–0 ^1L_b tryptophanyl CD band (290.8 nm) is displaced from the absorption maximum (289.4 nm) by the more intense negative CD band at 287.8 nm. Shifts due to multiple species or excitons can be ruled out, because the single tryptophanyl side chain of horse heart cytochrome c is buried in a single, well-defined site.[157]

The negative fine structure at 287.8 and 280.6 nm has the characteristics expected for tyrosyl CD bands overlapped by positive ^1L_b tryptophanyl bands. The impressive sharpness of the negative fine structure at 77°K resembles the tyrosyl CD bands in L-tyrosyl-L-tryptophanyl diketopiperazine at 77°K.[52] In contrast, ^1L_a CD bands of single tryptophanyl species do not show such impressive sharpening. Thus, both the 77°K spectra and Myer's chemical modification studies strongly suggest that the negative CD fine structure results from tyrosyl side chains. Apparently, the overlapping positive ^1L_b tryptophanyl CD bands (291 and 284 nm) greatly enhance the sharpness of the negative tyrosyl bands (281 and 288 nm).

Perhaps, the positive 0–0 ^1L_b tryptophanyl CD band may give rise to the positive CD fine structure observed at 291 to 293 nm in the room temperature spectra of ferricytochrome c isolated from turkey,[225] chicken, and turtle heart.[223] The latter proteins, which contain the same tryptophanyl and tyrosyl side chains as the horse protein, probably have nearly the same conformation as that reported for horse ferricytochrome c.[157]

Cytochrome b_5

Huntley and Strittmatter[177] have described the CD spectra of the oxidized form obtained using lipase extraction from rabbit and calf liver microsomes. Both proteins have one heme, one tryptophanyl, three phenylalanyls, and lack disulfide bonds.[164] Rabbit cytochrome b_5 has 3 tyrosyls (6, 7, and 30). The protein from calf liver has these tyrosyls plus an additional one at position 27.[227] Figure 30 shows that the near-UV CD spectra of these heme proteins are relatively similar. The sharpness and wavelength positions of the CD fine structure suggest that the ^1L_b tryptophanyl transition produces the CD bands at 286 ($0 + 850$ cm^{-1}) and 292 nm (0–0). The positive bands are superimposed upon a more intense negative CD extending from about 315 to 257 nm. There is also CD fine structure near 280 nm. Its origin, however, cannot be assigned solely on the basis of wavelength position.

Apocytochrome b_5 from calf has a CD spectrum whose shape resembles that of the holoprotein, even though the apo form has somewhat less negative CD between 300 and 260 nm.[177,227] Apparently, the aromatic amino acid side chains contribute a large part of the CD between 260 and 300 nm in oxidized cytochrome b_5. The ^1L_a tryptophanyl CD at 298 nm is relatively intense; apocytochrome b_5 has $\Delta\epsilon^{298}$ equal $-1.8\ M^{-1}\ cm^{-1}$. The ^1L_b tryptophanyl CD intensity in both apo and holo forms may be estimated from the height of the sharp ^1L_b band at 292 nm relative to the CD at 298 nm. These measurements indicate that removing the heme does not much alter the intensity of the 0–0 ^1L_b tryptophanyl band ($\Delta\epsilon^{292} - \Delta\epsilon^{298} \approx 1.2\ M^{-1}\ cm^{-1}$). This result is understandable in terms of the X-ray analysis of crystalline calf cytochrome b_5,[228] which shows that the tryptophanyl side chain is remote from the heme group (about 12 Å between closest transition monopoles). Thus, coupling between transitions in these two moieties is not likely to produce large near-UV CD, e.g., see Reference 39. In contrast, Tyr-30 seems close enough to the heme to have appreciable CD due to coupling with its transitions (see Table 10). Apparently, the difference in CD (300 to 260 nm) between the apo- and holoprotein may reflect heme CD, an altered conformation, and/or loss of coupling between tyrosyl and heme transitions.

Huntley and Strittmatter[177] have investigated the tyrosyl CD by acetylating calf and rabbit cytochrome b_5. All 3 of the tyrosyls in rabbit cytochrome b_5 and 3 of the 4 tyrosyls in the calf protein were reported to be acetylated at pH 7.5. This finding, together with sequence data, led Huntley and Strittmatter[227] to suggest that the extra tyrosyl of the calf protein (Tyr-27) had remained unacetylated. Acetylation of either protein caused a large loss of CD between 260 and 290 nm ($\Delta\Delta\epsilon^{268} \approx 2.6\ M^{-1}\ cm^{-1}$ in Figure 30).

When the *O*-acetyltyrosyls of rabbit cytochrome b_5 were deacetylated with hydroxylamine, about two thirds of the lost CD was recovered between 260 and 290 nm ($\Delta\Delta\epsilon^{268} = -1.9\ M^{-1}\ cm^{-1}$ in the right side of Figure 30). Presumably, the irreversible part represents the effects of acetylating groups other than the tyrosyl side chains. Below 270 nm, the reversible CD change may involve *O*-acetyltyrosyl, tyrosyl, ^1L_a tryptophanyl, and/or heme bands. The absence of fine structure between 260 and 270 nm, however, may perhaps indicate that the CD of the *O*-acetyltyrosyl side chains is small. Judging from the wavelength profile of the reversible CD changes, the alteration between 275 and 285 nm may reflect mainly the CD from tyrosyl side chains. Other groups may also contribute partially to the observed CD changes, since deacetylation seems to cause small CD changes even where tyrosyl does not have CD, e.g., above 300 nm (see Figure 30). These changes in nontyrosyl CD may involve an altered conformation or altered μ–μ coupling with other groups. Acetylating or deacetylating tyrosyls may indirectly change the CD of other nearby groups whose transitions couple with the tyrosyl transitions; e.g., the side chain of Tyr-7 is practically in contact with the side chain of Trp-22, and Tyr-30 is close to the heme (see Table 10).

TABLE 10

Separations Between Nearby Aromatic Rings in Calf Cytochrome b_5[a]

Tyr	Nearby Ring	r^b_{min}	R_{21}[c]
6	Tyr-7	5	9
7	Trp-22	2	6
	Tyr-6	5	9
27	none		
30	Phe-74	5	8
	Phe-58	5	8
	heme	6	11

[a] Calculated from data of Mathews and Argos.[228] Includes all aromatic amino acid side chains with transition monopole separations[42] less than 7 A from other aromatic amino acid side chains and less than 10 A from heme.

[b] r_{min}, separation between closest transition monopoles in A.

[c] R_{21}, distance between ring centers in A.

Hemoglobin I of *Chironomus th. thummi*

Wollmer and Buse[145] have examined the CD spectra of this insect hemoglobin, which contains three tryptophanyls and lacks disulfide bonds. The intense positive CD band of the ferrihemoglobin between 250 and 275 nm may correspond to the 260-nm heme band observed in other compounds[117] (see Figure 31). In contrast to this band, the CD fine structure occurs in both the apo and native hemoglobin. The negative fine structure at 269 and 262 nm may be assigned to phenylalanyl.

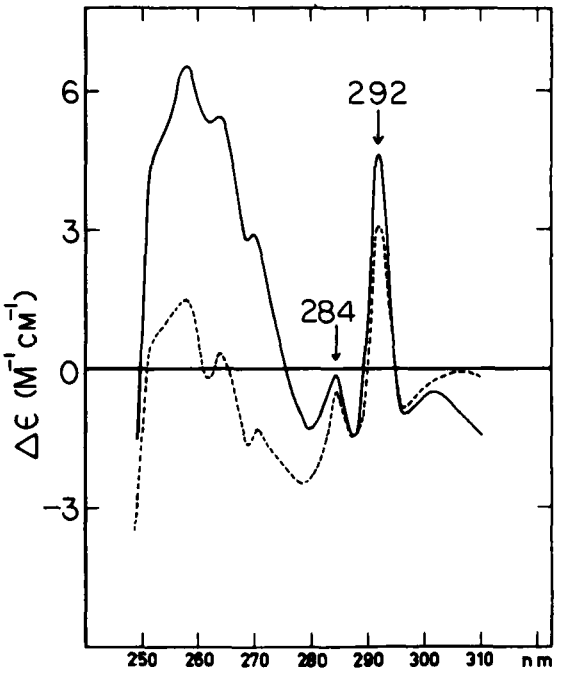

FIGURE 31. CD spectra of apo-(- - - -) and hemoglobin I from *Chironomus th. thummi*. Room temperature, pH 8.7. (From Wollmer and Base, *FEBS Lett.*, 16, 308, 1971. With permission.)

The intense, sharp positive CD band at 292 nm arises from the tryptophanyl side chains. Its sharpness and wavelength suggest it arises from the 0–0 ^1L_b transition.[145] A weaker, positive band at 284 nm has the correct spacing to be the 0 + 850 cm^{-1} ^1L_b tryptophanyl band. After correcting for the background CD, the ratio of heights of these two ^1L_b bands ($\Delta\epsilon^{0+850}/\Delta\epsilon^{0-0} = 0.2$) is less than half that observed for CD spectra of single tryptophanyl species (usually 0.6 to 0.8).[52] The smallness of this ratio and high intensity of the 0–0 band are consistent with an exciton interaction between ^1L_b transitions in neighboring tryptophanyl side chains. Wollmer[229] has presented evidence that a tryptophanyl-tryptophanyl interaction is indeed responsible for the 292-nm CD band of this insect hemoglobin. Based upon sequence data and analogy with a closely related insect hemoglobin whose conformation is known, he suggested that two tryptophanyl side chains are in proximity, possibly stacked.

Shethna Apoflavoprotein

The monomeric form prepared by Edmondson and Tollin[230] contains 4 tryptophanyls, 5 tyrosyls, 13 phenylalanyls, and lacks disulfide bonds. It has an intense ^1L_a tryptophanyl CD band at 297 nm and another intense band at 288 nm that probably also results mainly from tryptophanyl (see Figure 32).[230] This CD spectrum raises two intriguing questions. First, what interactions produce the intense ^1L_a tryptophanyl CD in the region above 285 nm? At 297 nm, the $\Delta\epsilon$ value per tryptophanyl is 2.9 $M^{-1}_{Trp}cm^{-1}$ ($\Delta\epsilon^{297}/\epsilon^{297} = 1.2 \times 10^{-3}$). Secondly, what causes the CD intensity to be so weak between 260 and 280 nm, where the ^1L_a tryptophanyl bands have their most intense absorption? One possibility is that an intense ^1L_a tryptophanyl exciton band is superimposed upon an intense positive ^1L_a tryptophanyl band of the single species type. Above 285 nm the positive exciton branch would intensify the positive CD and, at shorter wavelengths, the negative exciton branch would cancel the positive CD of the single species-type ^1L_a tryptophanyl band.

Hen Egg-White Lysozyme

CD spectra of this protein have been described in numerous publications.[169,175,198,231-235] The unusual interest in lysozyme resulted in part from the availability of its three-dimensional structure[189] and also from the marked changes in the near-UV CD bands following a variety of experimental manipulations.[169,175,186-188,231-241] Unfortunately, analysis of the near-UV CD of hen egg-white lysozyme is extremely difficult and has yet to be carried out rigorously. In the following paragraphs, I shall point out some of the problems presented by the lysozyme CD spectrum.

Figure 33 shows the CD spectra of hen egg-white lysozyme reported recently by Ikeda and Hamaguchi.[232] In agreement with earlier reports,[169,231,234] their spectra seem to have positive CD fine structure at 294, 288, and 282 nm and a strong negative region with a broad shoulder between 265 and 255 nm. At pH 7.2, Ikeda and Hamaguchi[232] also found weak negative CD at long wavelengths ($\Delta\epsilon^{305} = -0.13$ M^{-1} $cm.^{-1}$) which had not been seen previously: Subsequent to their publication, Strickland and Billups[76] confirmed the existence of this new negative CD above 300 nm. The negative CD at both the long and short wavelength ends undoubtedly is distorted by the positive CD bands (300-280 nm). Thus, the negative CD peaks do not necessarily represent actual band positions.

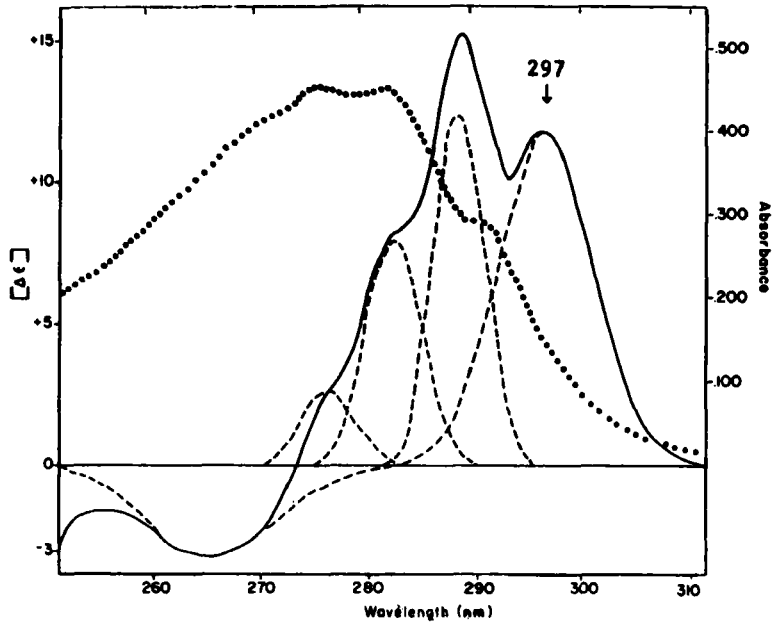

FIGURE 32. CD (–) and absorption (· · · ·) spectra of Shethna apoprotein. pH 7.0, room temperature. (Reprinted from Edmondson and Tollin, *Biochemistry*, 10, 127, 1971, © 1971 by the American Chemical Society. Reprinted by permission of the copyright owner.)

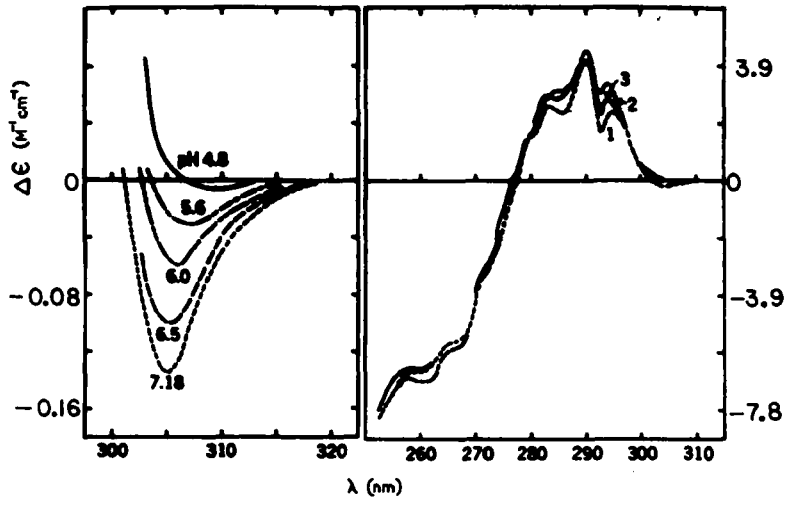

FIGURE 33. CD spectra of hen egg-white lysozyme at 298°K. On the right: 1, pH 7.2; 2, pH 4.2; 3, pH 1.2. (From Ikeda and Hamaguchi, *J. Biochem. (Tokyo)*, 71, 266, 1972. With permission.)

Possibly, a broad negative CD band extends throughout the near-UV but is hidden between 280 and 300 nm by more intense positive bands. Both regions of negative CD occur at wavelengths where ^1L_a tryptophanyl and disulfide may contribute CD.

The fine structure between 282 and 294 nm may be more complex than it appears at first glance. Even though the positive CD bands are dominant, both positive and negative fine structure bands may perhaps alternate within this region. The CD changes accompanying aggregation

TABLE 11

Separations Between Tryptophanyl Side Chains and Other Nearby Aromatic Rings in Hen Egg-White Lysozyme[a]

Trp	Nearby Ring	r^b_{min}	$R_{21}{}^c$
28	Trp-108	3	6
	Trp-111	5	8
	Tyr-23	3	7
	Tyr-20	4	7
62	Trp-63	3	7
63	Trp-62	3	7
	Trp-108	5	9
108	Trp-28	3	8
	Trp-111	4	8
	Trp-63	5	9
	Tyr-23	6	9
111	Trp-108	4	8
	Trp-28	5	8
	Tyr-23	2	6
123	Phe-34	3	6
	Phe-38	4	8

[a]Includes all aromatic side chains with transition monopole separations less than 7 Å. Calculated from preliminary set of coordinates supplied by Prof. D. C. Phillips in 1971.
[b]r_{min}, separation between closest monopoles in Å.
[c]R_{21}, separation between ring centers in Å.

of lysozyme may provide some support for this speculation. At high lysozyme concentrations (pH 7 to 8), the valleys at 285 and 292 nm become much more prominent.[239] In the monomeric lysozyme, the wavelength and rapid onset of the 294 nm positive CD band are indicative of a tryptophanyl contribution. If negative fine structure does exist at 292 nm, it also would be a tryptophanyl band. The wavelengths of the remaining fine structure (288 to 282 nm) are consistent with tryptophanyl and/or tyrosyl bands, e.g., see discussion of cytochrome c spectrum.

The complexity of the lysozyme CD spectra may be partially understood from the positions of the aromatic side chains reported for the crystalline state.[189] For didactic purposes, let us consider only μ–μ coupling between the six tryptophanyl, three tyrosyl, and three phenylalanyl side chains. With the exception of tryptophanyl-62, these rings are well-defined in the electron density maps,[189] suggesting that these side chains may be relatively rigid. Thus, when another aromatic ring is within 7 Å of a tryptophanyl side chain, appreciable CD intensity may be generated by μ–μ coupling, if the angles between transitions are favorable. Since tryptophanyl has two different transition directions in the near-UV, at least one of these transitions is likely to get some CD from each coupling interaction. Based upon the close separations shown in Table 11, one can estimate that at least 16 different interactions are likely to contribute part of the total tryptophanyl CD. By comparing this situation with that described for ribonuclease, one may begin to appreciate the enormously greater difficulty of interpreting the lysozyme CD spectrum in terms of either experimental or theoretical studies. Even when the transition moment directions and monopoles of tryptophanyl become well-established, theoretical calculations may not be sufficiently accurate to calculate the lysozyme CD spectrum.

Chemical modification of tyrosyl and tryptophanyl side chains has provided a basis for several unsubstantiated claims concerning the origin of the near-UV CD of lysozyme. Oxidation of Trp-108 with iodine has been reported to cause the CD to become negative throughout the near-UV and to eliminate the positive fine structure at 294 nm.[188] Initially, oxindolylalanyl was thought to be the product, but an ester of 2-hydroxytryptophan now seems more likely.[189] By assuming that the oxidized tryptophanyl side chain lacked optical activity in the 270-300 nm region, Teichberg et al.[188] concluded that the CD difference between native and oxidized lysozyme represented the CD of Trp-108.

Their conclusion is vitiated by two important observations. First, the oxidized tryptophanyl side chain may have appreciable CD throughout the near-UV.[193] Secondly, consideration must be given to the interactions between Trp-108, or its product, and other aromatic groups. In particular, Trp-108 is close to Trp-28, Trp-63, Trp-111, and Tyr-23 (see Table 11). Oxidizing Trp-108 may be expected to alter the intensities and directions of its transitions, which will affect μ–μ coupling with these other near-UV chromophores. Thus, modifying Trp-108 may also change the near-UV CD of Trp-28, Trp-63, Trp-111, and Tyr-23. This illustrates clearly that the CD effects of chemical modifications are not necessarily limited to a single side chain even if the modification is entirely specific for that residue. Evidently, the CD alteration upon oxidizing Trp-108 to either oxindolylalanine[189] or an ester of 2-hydroxy-

tryptophan[188] cannot provide a CD spectrum of the Trp-108 in native lysozyme.

By considering the CD characteristics of the oxidized tryptophanyl side chains, the CD spectra of modified lysozymes may perhaps indicate which residues contribute to the CD *fine structure* in the native protein. When oxindolylalanyl is the product, any CD fine structure probably reflects tryptophanyl or tyrosyl bands, because the oxindolylalanyl side chain has relatively broad CD bands.[193] In this regard, Trp-62 does not seem to contribute much to the fine structure between 282 and 294 nm, since its sharpness is little affected after oxidizing Trp-62 with *N*-bromosuccinimide.[186,187,237] The intensity of the broad ^1L_a band of Trp-62, of course, cannot be estimated by oxidation, because the product may also have broad CD bands in the near-UV. Oxidizing a second tryptophanyl with *N*-bromosuccinimide causes loss of the 294-nm fine structure but not the 288 and 282-nm fine structure.[187] Interpreting this latter observation must await a complete characterization of the products and should consider possible changes in CD of other tryptophanyls that couple with the modified side chain.

The negative 305-nm band of native lysozyme has been examined by Ikeda and Hamaguchi[232] by using pH variations between one and seven. The intensity of this band decreases to zero at pH 4, having a midpoint at about pH 6 (see Figure 33). This value corresponds approximately to the pK of Glu-35.[189,232] In view of the proximity of Glu-35 and Trp-108, Ikeda and Hamaguchi suggested that protonating Glu-35 alters the CD of Trp-108.

The effects of alkaline pH's upon the lysozyme CD spectrum have been examined by Ikeda and Hamaguchi[169] and by Halper et al.[234] The pK values for the 3 tyrosyls appear to be about 10 (Tyr-23), 11.5 (Tyr-20), and 12.5 (Tyr-53).[189] CD measurements in the far-UV suggest that the native conformation is disrupted as Tyr-53 becomes ionized.[169,189] Ionizing the first tyrosyl (23) produces an intense positive CD band at 298 nm ($\Delta\Delta\epsilon^{298} \approx 3\ M^{-1}cm^{-1}$), but ionizing the second (Tyr-20) does not much affect the CD at 298 nm.[169,234] The initial CD changes at long wavelengths may not be entirely due to the phenolate chromophore, because Tyr-23 is near Trp-28, Trp-108, and Trp-111 (see Table 11). Thus, ionizing Tyr-23 may alter its coupling with these tryptophanyl side chains, thereby also changing their CD.

Limited information about the tyrosyl CD bands has been obtained from acetylated lysozyme.[175] Acetylating about four amino groups and two tyrosyls (probably 20 and 23) alters CD intensities throughout the near-UV but does not eliminate the fine structure CD bands between 282 and 294 nm. Apparently, only one of the tyrosyls (53?) may possibly contribute substantially to this fine structure. The broad-band CD changes result partly from acetylating the two tyrosyls and partly from acetylating the four amino groups.[175]

In addition to the aromatic CD contributions, the four disulfide bridges of lysozyme may give near-UV CD.[198] Tamburro et al.[207] have published a low-resolution CD spectrum of lysozyme, having one disulfide bridge cleaved (Cys 6-127). This CD spectrum shows 10 to 30% intensity changes throughout the near-UV. The fully reduced lysozyme loses most of the near-UV CD, suggesting disruption of most tertiary structure.[207,242]

Recently, Barnes et al.[235] have implied that the negative CD plateau (265-255 nm) of the native lysozyme may arise primarily from disulfide bonds. They pointed out that CD changes in this plateau correlate with the increased reducibility of these disulfides in 8 *M* urea. Unfortunately, this evidence is inconclusive. Conformation changes affecting the accessibility of the disulfides may also alter the orientation of tryptophanyl side chains. With the exception of the disulfide bond connecting Cys-6 and Cys-127, all disulfide bonds are near at least one tryptophanyl side chain.[189]

Human Lysozyme

Halper et al.[234] and Ikeda et al.[243] have described the CD spectra of human lysozyme at neutral and alkaline pH's. Although interpreting these spectra is beset with many of the difficulties described for chicken lysozyme, one aspect of the human lysozyme CD spectra deserves comment here. Raising the pH to 10 produces a negative CD band that seems to peak about 313 nm. The growth of this band parallels ionization of the first three tyrosyl side chains ($\Delta\epsilon^{313} = -2.3\ M^{-1}cm^{-1}$ at pH 11.5).[234] Halper et al.[234] called this band "anomalous" in view of its long wavelength. These room temperature CD spectra, however, may not indicate the actual position of the underlying

transition, because the bands at shorter wavelengths may shift the apparent position. Both disulfide and tyrosinate moieties should be considered as possible sources of the 313-nm CD band in human lysozyme.

USES OF NEAR-UV CD SPECTRA IN PROTEIN CHEMISTRY AND ENZYMOLOGY

Near-UV CD measurements may sometimes be the most sensitive and convenient way to measure many of the routinely studied properties of proteins, e.g., enzymic activity,[68] binding constants,[68,143,169,186,237,238,241,244-246] denaturation or altered tertiary structure, nucleic acid contamination of proteins,[171] and interactions between molecules. Often, CD spectra provide information about these practical problems without needing a detailed spectral interpretation. Understanding the moieties giving CD, however, may permit extracting some conformational information. This section describes several applications of near-UV CD and points out potential pitfalls in their interpretation.

Detecting an Altered Protein Conformation

Near-UV CD spectra have been used to detect a variety of conformational changes or differences, e.g., comparing related proteins,[28,67,126,136,149,150,152-154,223,225,247-255] determining effects of deleting residues,[179,204,205,210,211,220,256] studying the reconstitution of a protein from its components,[205,257,258] identifying conformational role of disulfides[115,146,148,171,201,203,207,242,259,260] or other side chains,[261] establishing conditions for denaturation,[28,67,114,125,136,143,144,146,149,210,211,224,231,233,235,237,246,253,254,258,262-275] and determining temperature effects. When comparing modified proteins, one must consider the possibility that CD differences reflect a chromophoric modification rather than imply a major conformational difference. For example, converting the four disulfide bonds of ribonuclease-A to –S–Hg–S– bonds deletes the disulfide chromophore and adds the mercaptide chromophore.[208] Perhaps much of the CD change following this modification[208] reflects these chromophoric alterations.

The remainder of this subsection describes the effects of temperature and denaturing agents upon near-UV CD spectra.

Effects of temperature – Each protein may have conformers differing in their backbone or side chain orientations. Even in the native protein, several conformers may be in equilibrium, because thermal energy permits populating conformers having slightly more than the minimal free energy ($\log_e (N_1/N_2) = -\Delta G/RT$). Temperature changes can affect the distribution of protein conformers by altering both RT and the Gibbs free energy (ΔG) separating the various conformers. When the free energy difference between conformers changes rapidly within a narrow temperature range, one conformer or group of conformers may dominate at lower temperatures and another at higher temperatures, e.g., native and heat-denatured protein.

For qualitative purposes, conformational changes may be divided into two types: cooperative[276] (ΔG highly temperature-dependent) and noncooperative (ΔG relatively constant). A cooperative conformational change occurs over a narrow temperature interval (perhaps less than 20°C), giving rise to the so-called melting curve. In this case, both groups of conformers exist simultaneously only within a small temperature range. A noncooperative conformational change occurs gradually over a wide temperature range (more than 50°C), in which two or more conformers are in equilibrium. The existence of multiple conformers in equilibrium indicates that the conformers interconvert. Hence, noncooperative conformational changes indicate conformational motility.

CD offers a convenient method to determine if temperature changes affect the native conformation. A few near-UV CD spectra have been published of proteins at higher temperatures. With elastase,[277] ribonuclease-A and derivatives,[174,204,210,211,278,279] ribonuclease-T_1,[266] and chymotrypsinogen,[278] heating causes a major loss of near-UV CD intensity over a narrow temperature range (melting temperature usually less than 60°C). α-Gliadin shows a gradual loss of near-UV CD intensity between 30 and 60°C.[280] The loss of CD at high temperatures suggests an increased motility of the side chains in these proteins.

Cooling has also been reported to alter CD spectra of several proteins. Both pyruvate kinase[281] and the coat protein of a TMV mutant[247] have appreciably intensified near-UV CD spectra upon cooling only 20°C below room temperature. In addition, the wavelength profiles of these CD spectra are markedly altered,

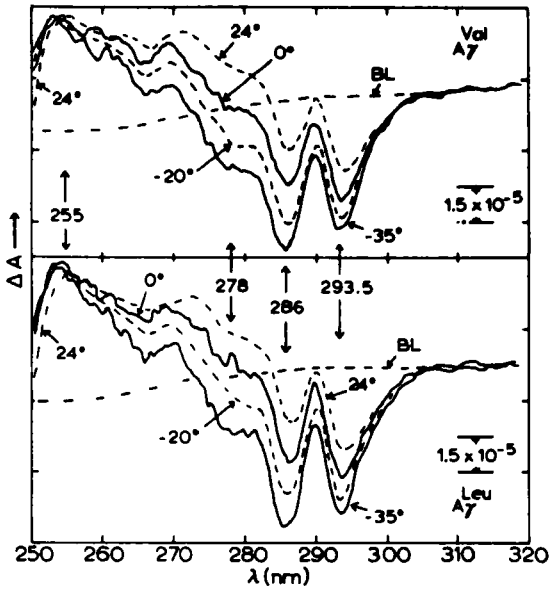

FIGURE 34. Variable low temperature CD spectra of carboxypeptidase-A_γ^{Val} (51.7 μM) and -A_γ^{Leu} (59 μM). Temperatures are listed in degrees centigrade. BL designates baseline. 2.0- mm path length. (Reproduced from Fretto and Strickland, *Biochim. Biophys. Acta*, 235, 482 (Figure 6), 1971. With permission.)

suggesting major changes in tertiary structure. To determine whether these are cooperative transitions, CD spectra need to be recorded at several additional temperatures.

Noncooperative conformational changes have been observed upon cooling several species of carboxypeptidase-A.[28] Cooling to 238°K gradually intensifies the negative CD above 275nm (see Figure 34). The area under the CD band between 290 and 310 nm, which arises from tryptophanyl side chains,[28,171] increases 1.9-fold upon cooling from 297 to 238°K. This enhanced CD does not seem to result from aggregation upon cooling, because similar results were obtained over a 200-fold concentration range. Apparently, cooling alters the orientations of tryptophanyl and perhaps other side chains of carboxypeptidase-A. Since the area under the long wavelength CD bands increases gradually as the temperature is lowered over a wide range, the cooling experiments indicate that the tryptophanyl side chains have some motility at room temperature. Interestingly, there is no measurable change in the far-UV CD spectrum (200-250 nm) caused by cooling carboxypeptidase-A to either 233°K or 77°K. Apparently cooling does not much affect the peptide backbone conformation.[28]

The rotatory strengths of ribonuclease-A and -S have also been examined at low temperature.[137] Cooling from 297 to 248 or 77°K causes less than a 10% increase in the area of the near-UV CD band above 250 nm, which arises mainly from the tyrosyl and cystinyl side chains. Thus, the ribonuclease CD spectra do not indicate any major motility of their tyrosyl or cystinyl side chains. This negative result, however, should be viewed with appropriate caution. Even some flexible tyrosyl derivatives do not show much CD enhancement upon cooling; e.g., the rotatory strength of L-tyrosine in water-glycerol increases only 33% upon cooling from 298 to 77°K.[49] (See also Reference 110.) The absence of a large change in rotatory strength upon cooling does not necessarily prove rigidity, because several conformers may have nearly the same rotatory strength. Furthermore, the relative populations of the conformers may not be shifted readily by temperature changes, especially in a highly interactive solvent such as one containing water.

Another complexity encountered with some proteins results from band sharpening upon cooling. When both positive and negative CD bands overlap, there is cancellation in the region of overlap. As the bands sharpen, cancellation becomes less extensive, producing an apparent increase in area under both the positive and negative CD bands. The actual change in rotatory strength can be obtained only after correcting for the difference in overlap at the two temperatures. This effect is clearly evident in the tryptophanyl CD fine structure of chymotrypsinogen-A cooled to 77°K.[24] A similar cancellation probably occurs in the 240-nm positive CD band of ribonuclease, which is overlapped on both sides by negative CD bands. Whether band sharpening can account for the temperature sensitivity of the 240-nm CD band of ribonuclease[179,204] appears not to have been investigated.

Effects of denaturing agents – CD spectra provide a way to measure the extent of structure remaining after treating a protein with denaturing agents. Ideally, the near-UV CD intensities should permit determining whether the aromatic amino acid side chains become fully motile in various denatured states. Several practical difficulties tend to impede a complete analysis of CD spectra belonging to denatured proteins, although none is

insurmountable. First, the CD intensities of denatured proteins are small and can be measured accurately only with a high-performance CD instrument. Secondly, the CD bands of any disulfide bonds may tend to mask the weak CD bands of motile aromatic amino acid side chains. Thirdly, more extensive studies of denatured proteins are needed to determine the range of CD intensities expected from motile phenylalanyl, tyrosyl, and tryptophanyl side chains.

Until these data become available, the most appropriate model compounds seem to be the *N*-acetyl amides of tyrosine, phenylalanine, and tryptophan dissolved in 8 *M* guanidine hydrochloride (see Tables 2 to 4). In a fully denatured protein lacking disulfide bonds, the $\Delta\epsilon$ value would be expected to be about $-0.05\ M^{-1}\ cm^{-1}$ (268-nm fine structure) for each phenylalanyl, -0.15 (275 nm) per tyrosyl, and 0.2 (290 nm) per tryptophanyl. Most likely, the CD signs of motile side chains in a fully denatured protein would be the same as those of the *N*-acetyl amino acid amides dissolved in guanidine hydrochloride. Even limited interactions of an aromatic ring with neighboring moieties, however, might possibly be sufficient to affect its CD sign.

For proteins containing only a few aromatic amino acid side chains, the native protein is likely to have much more intense CD than the denatured protein. Thus, a large loss of near-UV CD intensity is observed upon treating many proteins with guanidine hydrochloride or sodium dodecyl sulfate (SDS), e.g., lysozyme,[231,237] horseradish peroxidase,[67] ribonuclease-A and derivatives,[210,211] carbonic anhydrase,[125,254] and ovine pituitary lactogenic hormone.[149]

Soybean trypsin inhibitor (Kunitz), however, gives a different result. Even though the protein has an intense near-UV CD band relative to its amino acid content (two Trp, four Tyr, two disulfides),[282] 6 *M* guanidine hydrochloride causes only a 20% loss of near-UV CD.[283] In contrast, SDS decreases $\Delta\epsilon$ at 275 nm from -10 $M^{-1}cm^{-1}$ to $-0.8\ M^{-1}cm^{-1}$,* even though the inhibitor becomes more helical.[284] Apparently, SDS effectively disrupts the tertiary structure of soybean trypsin inhibitor, even though 6 *M* guanidine hydrochloride does not.

When a protein contains numerous aromatic side chains of a single kind, the possible effects of denaturing agents become more complex. With oriented side chains, the average $\Delta\epsilon$ per tryptophanyl, per tyrosyl, or per phenylalanyl will tend to decrease as the number of that particular side chain increases.[67] Different statistical conditions exist in a fully denatured protein. When the side chains are highly motile, each side chain of a single type may be anticipated to have nearly the same CD intensity and sign. Even though each motile side chain has weak CD, no cancellation occurs upon adding the CD spectra for all side chains of that kind. Thus, the total CD for tyrosyl, tryptophanyl, or phenylalanyl of a denatured protein may be expected to be proportional to the number of that particular side chain.

Horseradish peroxidase-C provides an example of cancellation of CD intensity in a native protein.[67] The total CD arising from its 23 phenylalanyl side chains is zero in the native conformation. Treating peroxidase-C with 8 *M* guanidine hydrochloride abolishes most of the near-UV CD intensity but brings out the characteristic phenylalanyl CD fine structure at 268 and 261 nm. Its intensity per phenylalanyl residue is comparable to that observed for Ac-Phe-NH_2 in guanidine hydrochloride. Apparently, guanidine hydrochloride increases the motility of the phenylalanyl side chains of peroxidase-C, thereby increasing the total phenylalanyl CD intensity.

Denaturing agents produce even more complex changes in the CD spectrum of a κ-type immunoglobulin (see Figure 35),[285] which has about 45 tryptophanyls, 56 tyrosyls, 45 phenylalanyls, and 22 disulfide bonds among its 1,615 residues.[286] In the native immunoglobulin (curve one, in Figure 35), the most intense CD bands are the positive fine structure at 290 and 283 nm. To obtain the largest possible average CD intensity, let us assume that the 290-nm CD band results only from the tryptophanyl side chains. Then the average $\Delta\epsilon^{290}$ per tryptophanyl is $0.3\ M_{Trp}^{-1}\ cm^{-1}$. Although this value is not much larger than that expected for motile side chains, it is more reasonable to assume that the aromatic side chains are oriented in the native immunoglobulin. Probably, the weakness of the average tryptophanyl CD results from extensive cancellation in summing tryptophanyl CD contributions having opposite signs. Adding 0.002 *M* SDS causes the CD to become more positive throughout the near-UV (see curve two in Figure

*-0.8 value based upon personal communication from B. Jirgensons.

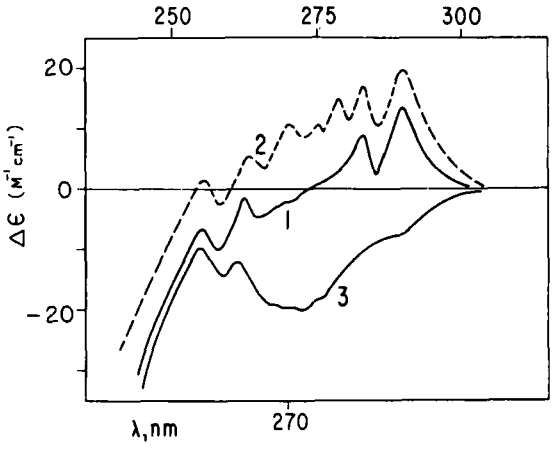

FIGURE 35. Effect of SDS upon CD spectrum of κ-type immunoglobulin. 1, native; 2, 0.002 *M* SDS; 3, 0.05 *M* SDS. pH 6.3, room temperature. (Reproduced from Jirgensons, *Biochim. Biophys. Acta*, 317, 134 (Figure 3), 1973. With permission.)

35). At a higher SDS concentration (0.05 *M*), the near-UV CD becomes more negative, and the fine structure at 290 and 283 nm largely disappears (see curve three, in Figure 35). The near-UV CD spectrum in 0.05 *M* SDS is also similar to that observed in 6 *M* guanidine hydrochloride.[285] The latter observation tends to suggest that curve three represents the immunoglobulin with relatively motile aromatic side chains. This question, however, is difficult to evaluate solely from the CD intensity, because the 22 disulfide bonds may also contribute CD.

In some ways, the effects of SDS upon proteins[200,277,284] resemble the changes caused by the helix-inducing organic solvents such as 2-chloroethanol. The mechanism by which the latter compound affects the conformation of proteins has been illustrated by Timasheff,[236] using lysozyme. Its near-UV CD spectra indicate that at low concentrations (12.5 volume %) 2-chloroethanol interacts with the aromatic side chains without disordering them. Higher concentrations of 2-chloroethanol (20%) greatly decrease the near-UV CD intensity and begin to increase the far-UV CD intensity. At 80% 2-chloroethanol, the 220-nm CD intensity is twice that observed in the native lysozyme. Evidently, high concentrations of 2-chloroethanol increase the motility of the aromatic side chains and also increase the helical content of lysozyme.[186,236]

Interactions Between Molecules

Numerous interactions have been reported to cause readily measurable changes in the near-UV CD bands of proteins. Sometimes these CD alterations reflect changes in tertiary structure. Unfortunately, the tendency has been to attribute conformational significance to any CD change accompanying interactions between a protein and another molecule (an effector). Such a conclusion is unwarranted without a more rigorous spectral analysis than has often been made.

Relating CD changes to an altered protein conformation is difficult, because one must distinguish among four possible explanations for the CD changes: (a) The protein conformation may be altered when the effector is bound; (b) The effector conformation may be different in the bound state; (c) Local interactions at the binding site may induce new CD bands or alter existing CD bands in either the protein or the effector even though no conformation change occurs; and (d) Long-range interactions between chromophores of the protein and those of the effector (μ–μ or μ–m coupling) may alter CD intensities of both the protein and the effector even in the absence of a conformation change. Distinguishing among these four interpretations may be aided by understanding the spectral properties of the effector. Interactions with effectors lacking near-UV bands are usually more easily interpreted. Changes in aromatic CD fine structure sizes may facilitate showing that the CD bands belong to the protein. Considering the size of the CD changes may indicate how many side chains must have altered CD bands. If the three-dimensional structure of the protein is known in the crystalline state, it may facilitate interpreting CD alterations in terms of specific side chains.

Ion binding – The near-UV CD spectra of some proteins undergo large changes upon binding certain cations.[143,248,281,287-290] Several of these proteins will be used to illustrate interpreting the CD changes accompanying ion binding.

CD changes induced by ions lacking any near-UV absorption, e.g., K^+ and Ca^{++}, can be attributed to protein CD bands. Nevertheless, an altered CD spectrum does not necessarily imply a conformation change, especially if the binding site has moieties giving near-UV CD bands. Several ways exist for these ions to alter CD bands even without changing protein conformation. First, if an ion binds within several Å of aromatic moieties, its

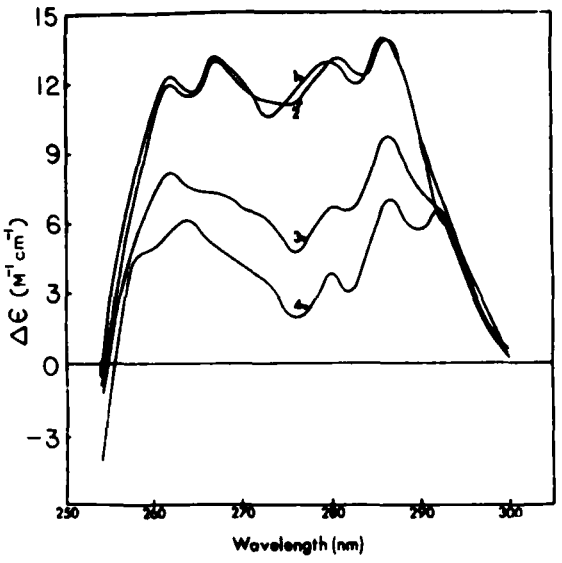

FIGURE 36. Effect of KCl (1), LiCl (2) or tetramethylammonium chloride (3) upon CD spectrum of rabbit muscle pyruvate kinase dissolved in tricine buffer (4). pH 7.4, 298°K. Cation concentrations were 0.1 *M*. (Reproduced from Wildes et al.,[281] *Biochim. Biophys. Acta*, 229, 852 (Figure 2), 1971. With permission).

electric field may possibly induce aromatic CD bands by the static perturbation mechanism (see Table 1). Although this CD effect may be weak, it needs to be investigated more fully. Second, the vacuum-UV absorption bands of the bound ion may couple with the near-UV bands of aromatic side chains. The large energy separation between these bands, however, suggests that this coupling may be relatively weak. Third, if a cation binds to a tyrosyl side chain, its hydroxy group may become ionized.[291] Thus, the cation binding may convert a tyrosyl CD band into a tyrosinate-like CD band.[289] This ionization may also alter coupling between that tyrosyl side chain and any neighboring aromatic side chains. Another complication is that ion binding near a disulfide bridge may perhaps alter its CD bands even without a conformation change.

Wildes et al.[281] examined the effects of K^+ and Li^+ upon the CD spectrum of pyruvate kinase, which has 4 monovalent cation-binding sites,[292] 12 Trp, 38 Tyr, 65 Phe, and possibly no disulfide bonds distributed among 4 similar subunits.[293] Neither K^+ nor Li^+ affects the far-UV CD. Either one causes major changes in the near-UV CD intensity and wavelength profile (see Figure 36). Although the change in CD intensity per binding site is 2.4 M^{-1} cm^{-1} at 276 nm, this value is not sufficiently large to prove that more than one side chain must have had its CD bands altered (see Table 6). The changes in wavelength profile do suggest that phenylalanyl, tryptophanyl, and tyrosyl CD bands may be affected by K^+- or Li^+-binding. For the most part, these CD changes seem to reflect an altered tertiary structure, because cooling from 298 to 278°K causes CD changes similar to those shown for K^+ or Li^+.[281]

Bovine trypsin is another enzyme showing a marked change in the near-UV CD spectrum upon binding a nonabsorbing cation. Ishida et al.[287] reported that 20 m*M* Ca^{++} intensifies the major near-UV CD band of trypsin at pH 8.2 (change in $\Delta\epsilon^{275} = -6.4$ M^{-1} cm^{-1}). In addition, Ca^{++} binding brings out negative CD fine structure at about 285 nm. Since each trypsin molecule is thought to bind only one Ca^{++} firmly,[294] the change in CD intensity per binding site seems relatively large (-6.4 M^{-1} cm^{-1}). Although a change of this size might possibly result from an intense ^1L_a tryptophanyl band changing sign upon Ca^{++} binding (see Table 6), it seems more likely that the CD bands of several side chains are enhanced. Perhaps in the future these CD changes can be interpreted in more detail by using the structural information now becoming available on crystalline trypsin.[294] Stroud et al.[294] have suggested that the carboxylate side chains of Asp-71 and either Glu-77 or Asp-153 may form the Ca^{++}-binding site in a region outside the active site. If subsequent studies confirm that aromatic and disulfide moieties are absent from the binding site, then the large CD change suggests that Ca^{++} binding may alter the tertiary structure of several side chains having near-UV CD bands.

The binding of cations by apotransferrin has also been examined using CD.[289,290] The single peptide chain of human serum transferrin has two high-affinity binding sites for cations such as Fe^{+++}, Cu^{++}, and Zn^{++}. Nagy and Lehrer[289] wisely used Zn^{++} for most of their near-UV studies, because bound Zn^{++} lacks absorption and CD above 270 nm (see Other Groups Which May Have Near-UV CD). Changes in the absorption spectrum upon binding two Zn^{++} suggest that two tyrosyl hydroxy groups become ionized. Apparently, one ligand of each Zn^{++} may be a tyrosinate side chain.[289]

Zn^{++} binding also causes an appreciable change in the near-UV CD spectrum. Nagy and Lehrer[289] examined this alteration in more detail by calcu-

lating the difference CD spectrum: Zn^{++}-transferrin minus apotransferrin. This spectrum had a broad negative maximum at 292 nm ($\Delta\Delta\epsilon^{292} = -17 M^{-1}$ cm^{-1}) resembling the tyrosinate absorption band. Thus, Nagy and Lehrer suggested that the difference CD spectrum arose from the two tyrosinate side chains interacting with Zn^{++}. Although this suggestion seems qualitatively consistent with their observations, it is not quantitatively in agreement with the CD characteristics of tyrosinate side chains. The difference CD intensity at 292 nm is $-8.5 M^{-1}$ cm^{-1} per tyrosinate, an extremely large value. Zn^{++} bound to the phenolate oxygen atom is not likely to induce much CD by static perturbation, because the Zn^{++} would be located in or near both symmetry planes of the aromatic ring (see Table 1). Thus, the tyrosinate CD intensity is not likely to greatly exceed that observed for the model compounds described in Table 7. Even if the tyrosyl CD is initially large and changes sign upon Zn^{++} binding, the change in $\Delta\epsilon$ is not likely to exceed $-4 M^{-1}$ cm^{-1} per tyrosinate. Apparently, more than two side chains have altered near-UV CD bands after Zn^{++} binding to transferrin.

The near-UV CD spectrum of Cu^{++}-transferrin was reported by Nagy and Lehrer[289] to be essentially identical to that observed for Zn^{++}-transferrin. Apparently, Cu^{++} itself does not have any strong near-UV CD bands in this particular complex. Tomimatsu and Vickery[290] have also reported the near-UV CD spectra of Cu^{++} and apotransferrin. Their difference CD spectrum for these forms, however, peaks at 289 nm and has a shoulder at 295 nm. Only this shoulder has the wavelength expected for a tyrosinate CD band. Evidently, tyrosinate is not the only moiety contributing to the difference CD spectrum for cation binding by transferrin.

Binding substrates or analogs lacking near-UV absorption – Near-UV CD changes accompanying binding of nonabsorbing substrates or analogs have been reported for homogeneous antibodies,[249] aspartate transcarbamylase,[129] aspartokinase-homoserine dehydrogenase,[143] carboxypeptidase-A,[68] concanavalin-A,[295,296] fumarase,[147] human[234,243] and hen egg-white lysozyme,[169, 188,231,232] and pyruvate kinase.[281] The interactions involving carboxypeptidase-A, hen egg-white lysozyme, and aspartate transcarbamylase will be examined in some detail.

Carboxypeptidase-A: X-ray diffraction studies showed that the active site tyrosyl of carboxypeptidase-A moves to a new position upon substrate binding. As the substrate Gly-Tyr is bound to crystalline carboxypeptidase-A, the side chain of Tyr-248 rotates by 120° about its C_α–C_β bond, moving its phenolic hydroxy group by 12 Å into the vicinity of the peptide bond on the substrate.[297] Fretto and Strickland[68] used CD to try to detect this conformational change upon binding the substrates Gly-Leu and Gly-Phe. Neither, however, measurably changed the tyrosyl CD bands of carboxypeptidase-A at 297 or 77°K (change in $\Delta\epsilon^{277}$ less than $\pm$ 0.3 M^{-1} cm^{-1} at 297°K).

In view of this negative experimental result, Fretto[298] examined theoretically whether the movement described for Tyr-248 would give a measurable CD change. The coordinates of crystalline carboxypeptidase-A and its complex with Gly-Tyr were used to calculate approximately the change in CD due to altered μ–μ coupling upon binding Gly-Leu (see Table 12). The movement of Tyr-248 per se alters several interactions giving near-UV tyrosyl CD. The largest two involve Tyr-248 coupling with the peptide bonds of carboxypeptidase-A and with other tyrosyl rings. Either of these CD changes alone would be measurable ($\Delta\Delta\epsilon^{max} \approx -0.65 M^{-1}$ cm^{-1} each). In addition, positive CD alterations are predicted. For example, the existence of a peptide bond in the substrate gives rise to an interaction with Tyr-248 that induces a + 0.55 M^{-1} cm^{-1} tyrosyl CD band (see Table 12). When all interactions are totaled, the calculated change in tyrosyl CD is less than 0.2 M^{-1} cm^{-1}, an unmeasurable change. Thus, even though the movement of Tyr-248 is large, the near-UV tyrosyl CD changes from the various μ–μ coupling interactions effectively cancel each other.

In contrast, the near-UV CD spectra of carboxypeptidase-A do permit measuring the binding of β-phenylpropionate,[68] an inhibitor which binds at both the active site and a second site within the pocket near Tyr-198.[297] Binding at the latter site causes a change in $\Delta\epsilon^{277}$ of about 2.5 M^{-1} cm^{-1} The difference CD spectra for β-phenylpropionate binding seem to reflect mainly tyrosyl CD, although a small tryptophanyl contribution is also evident.[68]

Hen egg-white lysozyme: The structure of lysozyme complexed with α- and β-*N*-acetylglucosamine and tri-*N*-acetylchitotriose has been determined for the crystalline state.[189] Both

TABLE 12

Calculated Change in Near-UV Tyrosyl CD of Carboxypeptidase-A_α Upon Binding of Gly-Leu and Resulting Movement of Tyr-248

(Based upon μ–μ coupling)[a]

^1L_b μ of	Coupling with μ of	$\Delta\Delta\epsilon^{max}$[b] M^{-1} cm^{-1}
Altered Interactions between Moieties of Carboxypeptidase-A		
Tyr-248	other Tyr rings	−0.65
Tyr-248	Phe rings	0.35
Tyr-248	His rings	−0.3
Tyr-248	peptide bonds	−0.65
Tyr-248	nearby side chain -COO$^-$	0.2
Tyr-248	nearby side chain amides	0.05
Tyr-248	Trp rings	~0[c]
Tyr-248	Arg side chains	d
other Tyr	Tyr-248 ring	0.4
Added Interactions between the Substrate and Carboxypeptidase-A		
Tyr-248'[e]	Substrate peptide bond	0.55
Tyr-248'[e]	Substrate -COO$^-$	0.1
other Tyr	Substrate peptide bond	0.1
other Tyr	Substrate -COO$^-$	~0
Total Change in Tyr ^1L_b CD		0.15

[a]Modified from Fretto.[298]

[b]The change in $\Delta\epsilon$, defined as the $\Delta\epsilon$ of interactions in the enzyme-substrate complex *minus* that of the corresponding interactions in the native enzyme.

[c]Since the closest Trp rings are more than 15 Å (center to center) away from Tyr-248 ring in either enzyme or enzyme-substrate complex, change in coupling with Trp is probably insignificant for binding Gly-Leu.

[d]Coupling with the nearby Arg-145 and Arg-127 may be significant, but these interactions cannot be calculated with sufficient accuracy to warrant their inclusion.

[e]Tyr-248' designates position of Tyr-248 in the enzyme-substrate complex.

β-*N*-acetylglucosamine and tri-*N*-acetylchitotriose form hydrogen bonds with indolyl >NH of Trp-62 and Trp-63. This interaction causes a small movement of Trp-62 and may increase its rigidity. In addition, the methyl group of an acetamido side chain is in contact with the ring of Trp-108.[299] α-*N*-acetylglucosamine binds next to Trp-108 but does not form a hydrogen bond with its ring.[189] On the basis of these extensive contacts between *N*-acetylglucosamine compounds and tryptophanyl side chains, one would anticipate that tryptophanyl CD bands may be altered upon binding.

Glazer and Simmons[231] showed some years ago that binding a mixture of α- and β-*N*-acetylglucosamine gives a 50 to 100% increase in the near-UV CD of lysozyme. A lower noise CD spectrum has been presented recently by Ikeda and Hamaguchi (see Figure 37).[232] Various *N*-acetylglucosamine derivatives enhance the lysozyme CD between 300 and 255 nm; e.g., at 294 nm β-methyl-*N*-acetylglucosamine increases the $\Delta\epsilon$ value by 2.7 M^{-1} cm^{-1}. The weak negative CD band of lysozyme at 305 nm is affected differently by each saccharide (see Figure 37). Adding *N*-acetylglucosamine weakens this band and shifts its apparent position to about 307 nm, whereas other saccharides increase the absolute size of this band (change in $\Delta\epsilon^{305}$ about 0.04 M^{-1} cm^{-1}). Perhaps these long wavelength CD changes reflect partly intensity alterations and partly wavelength shifts of ^1L_a tryptophanyl bands caused by saccharide binding. In this regard, note that the position of the ^1L_a tryptophanyl band is easily shifted by polar groups and by altered hydrogen bonding to its indolyl >NH (see Aromatic CD Bands in Model Compounds).

Binding effects have been examined most extensively in the region from 255 to 300 nm. Alcohols,[186,236] glycols,[186,231] 8 *M* urea,[235,237,275] and 2 *M* guanidine hydrochloride[237] have been reported to enhance CD in the region where tryptophanyl bands may occur. Apparently, the wavelength profiles of the various difference CD spectra (enzyme-effector complex minus enzyme) are not identical, but some of the variation may result from experimental uncertainty (see Figure 8 in Reference 169, Figure 2 in Reference 186, and Figure 8 in Reference 237).

Oxidizing Trp-62 or Trp-108 of lysozyme has been used in attempts to estimate which side chains contribute to the CD increase (255-300 nm) upon saccharide binding.[186,188,189] Modifying either Trp-62 or Trp-108 greatly diminishes the CD enhancement upon binding *N*-acetylglucosamine or tri-*N*-acetylchitotriose. These observations, plus the locations of Trp-62, -63, and -108 in the saccharide binding sites, suggest that all 3 tryptophanyl side chains probably contribute part of the CD increase (255-300 nm) upon binding β-*N*-acetylglucosamine residues to lysozyme.

Aspartate transcarbamylase from *Escherichia coli*: This allosteric enzyme is composed of six catalytic and six regulatory subunits. They can be separated from each other, and their CD spectra have been described.[128,129] There are no disul-

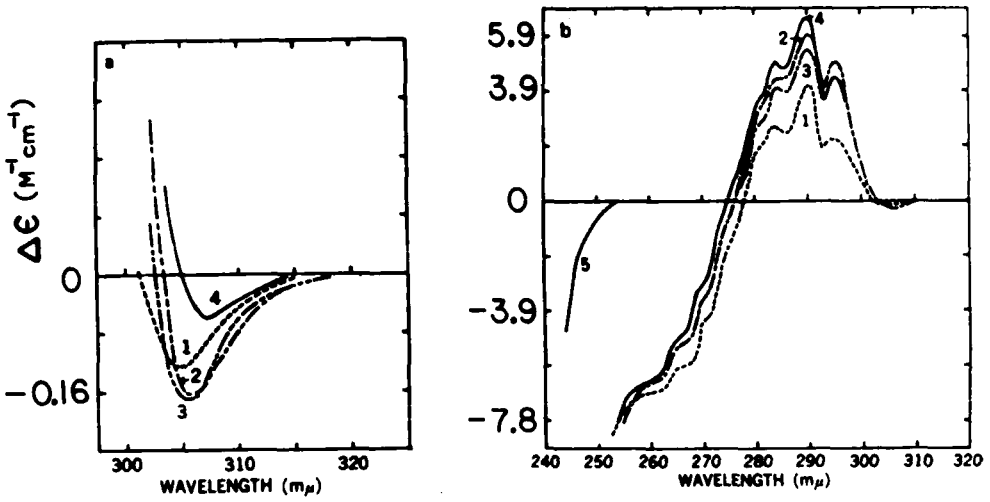

FIGURE 37. Effects of *N*-acetylglucosamine (4) and its α-(3) and β-methyl-glycosides (2) on the lysozyme CD spectrum (1). 5 is CD of *N*-acetylglucosamine. 298°K, pH 7.3. (From Ikeda and Hamaguchi, *J. Biochem. (Tokyo)*, 71, 270, 1972. With permission.)

fide bonds in this enzyme. Each regulatory subunit lacks tryptophanyl and has three tyrosyls, whereas each catalytic subunit has two tryptophanyls and nine tyrosyls.

Griffin, Rosenbusch, Weber, and Blout[129] have carried out an especially impressive analysis of the CD changes accompanying the binding of a substrate and a substrate analogue to aspartate transcarbamylase. Their study illustrates clearly the dividends reaped from a thorough analysis of CD spectra. Their results were presented in terms of difference CD spectra to facilitate a wavelength profile analysis. Additions of carbamyl phosphate and succinate were made to both the isolated catalytic subunit and native enzyme. These compounds interact cooperatively with the native enzyme but noncooperatively with the isolated catalytic subunit.

The difference CD spectra resulting from binding carbamyl phosphate are given by the two upper curves of Figure 38a (▲, aspartate transcarbamylase; ●, isolated catalytic subunits). These difference spectra have identical positive regions from 292 to 310 nm. Since these polypeptides lack disulfide, the positive CD above 300 nm reflects a more positive ^1L_a tryptophanyl CD in both the isolated and combined catalytic subunits, upon adding carbamyl phosphate. The difference CD spectrum for the isolated catalytic subunit has negative fine structure at 288 and 282 nm. Their shapes and wavelengths suggest an altered ^1L_b tryptophanyl CD in the isolated catalytic subunit, although this identification is not conclusive (see Analysis of cytochrome c spectrum). Adding carbamyl phosphate to the native enzyme causes a large positive CD enhancement peaking at 279 nm ($\Delta\Delta\epsilon \approx 2.2\ M^{-1}\ cm^{-1}$ per binding site) and having shoulders at 285 and 270 nm. The shape of this difference CD spectrum indicates that a large increase in tyrosyl CD occurs upon binding carbamyl phosphate to the native aspartate transcarbamylase.

Griffin et al.[129] further analyzed these difference CD spectra by subtracting the curve of the isolated catalytic subunit from that of the native enzyme (see Figure 38b). This difference difference CD spectrum, which may reflect only CD changes related to cooperative interactions, has the shape expected for tyrosyl CD bands without any obvious tryptophanyl contribution. The occurrence of the 0–0 band at 286 nm (0 + 800 band ~ 279 nm) suggests that the affected tyrosyl residues are located in a somewhat hydrophobic environment.[129]

Based upon these and other studies, Griffin et al.[129] suggested that tyrosyl residues may be involved in the stereochemical mechanism of allosteric interactions in aspartate transcarbamylase. Apparently, the tryptophanyl CD changes are inherent features of carbamyl phosphate binding in the presence or absence of allosteric

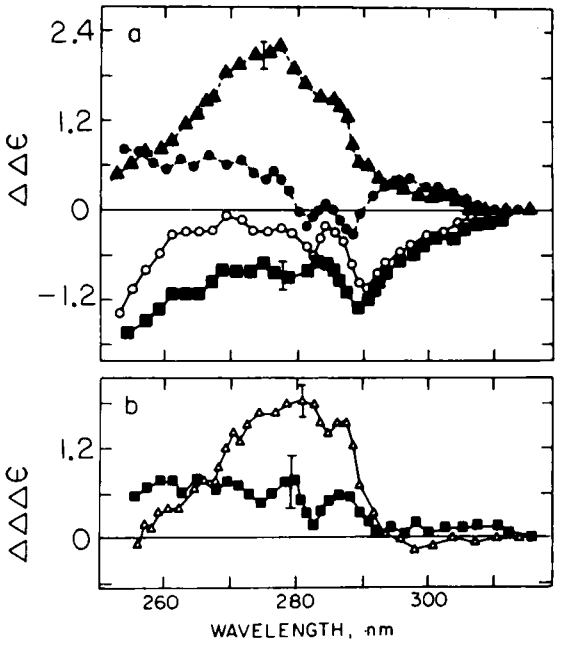

FIGURE 38. CD changes upon binding ligands either to aspartate transcarbamylase or to isolated catalytic subunit. 299°K, pH 7.0. CD intensity expressed relative to molar concentration of catalytic sites. (Top). Change in CD spectrum after carbamyl phosphate binding to aspartate transcarbamylase (▲) or to isolated catalytic subunit (●); change in CD after succinate binding in presence of carbamyl phosphate to aspartate transcarbamylase (○) or isolated catalytic subunit (■). (Bottom). Change in CD spectrum for binding to isolated catalytic subunit subtracted from that obtained for binding to aspartate transcarbamylase. △, carbamyl phosphate binding; ■, succinate binding. (From Griffin et al.,[129] *J. Biol. Chem.*, 247,6485, 1972. With permission.)

interactions and may indicate a tryptophanyl side chain in the active site region.

The CD changes upon adding succinate to isolated catalytic subunits and the native enzyme in presence of carbamyl phosphate were also reported.[129] Adding succinate perturbs tryptophanyl CD bands in both cases (see bottom curves of Figure 38a). The difference between these two difference CD spectra has a peak value of only 0.8 M^{-1} cm^{-1} at 279 nm, and its shape differs greatly from that described for carbamyl phosphate (see Figure 38b). The origin of the succinate difference difference CD spectrum needs further study.

Binding substrates, analogs, or cofactors having near-UV absorption – In these CD studies, it is often difficult to distinguish between the CD bands of the enzyme and those of the bound molecule. Furthermore, the possibility of coupling between chromophores of the protein and those of the effector may preclude obtaining any conformational information.

Nucleotide binding studies[129,174,244,245,266,300-302] are especially difficult to interpret. The relatively intense near-UV absorption of nucleotides permits extensive μ–μ coupling with protein moieties up to 10 or 15 Å away from the nucleotide. These interactions may alter the CD bands of aromatic side chains even without a conformation change and may also greatly alter the CD bands of the bound nucleotide.

An impressive example of μ–μ coupling has been described by Kägi et al.[303] Binding ADP to creatine phosphokinase induces a large positive CD band at 262 nm (change in $\Delta\epsilon$ = 21 M^{-1}cm^{-1}). This band, which arises from 2 mol of bound ADP, is 20 times more intense than that of unbound ADP and is 4 times more intense than the near-UV CD band of uncomplexed creatine phosphokinase. Kägi et al.[303] suggested that the large adenine CD band could be accounted for by coupling between the far-UV tryptophanyl bands and the near-UV adenine band.

Interactions between peptides, proteins, or subunits – Mixing two components often gives nonadditive CD changes in the near-UV, e.g., reassociation of the α and β subunits of luteinizing hormone (see Figure 25)[116] and of thyrotropin,[304] self-association of glucagon,[305] and many other intermolecular interactions.[106,107,129,150,154,239,272,306-309] These CD alterations may reflect conformational changes and/or coupling between groups on the two peptides. Intuitively, one expects that the near-UV CD is not likely to be additive when the region of contact has aromatic side chains, especially when the interaction brings together two or more aromatic side chains. However, interpreting CD changes in terms of conformation is difficult, because μ–μ coupling permits long-range interactions, and because conformation changes may occur both within and outside the region of contact.

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