Circular Dichroism of Proteins in Solution and at Interfaces

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I. OUTLINE

Compared with circular dichroism (CD) investigations of proteins in solution, rather few CD studies have been made of proteins localized at interfaces. The majority of the systems studied consist of proteins (or peptides) either adsorbed to or incorporated into artificial biological membranes. Such systems can be considered as liquid-liquid interfaces with which proteins are in one way or another associated. The even more scarcely studied category comprises proteins adsorbed at microscopic solid interfaces that are dispersed in aqueous solution. With respect to both situations, the question arises of whether the protein retains or changes its conformation upon interaction with the interfaces. Answers can be obtained by using CD spectroscopy as illustrated in this chapter.

In order to understand the use of CD spectroscopy applied to the conformational analysis of proteins, some theory has to be given first. The basic aspects of the technique as treated in numerous physicochemical textbooks (e.g., 1–3) will be summarized first. Books that provide an updated treatise on a variety of applications of circular dichroism (4,5) are also consulted. They give an in-depth survey of various aspects of circular dichroism as applied to biological macromolecules that are not (or are hardly) covered in this chapter. After the basic introduction, a section is devoted to secondary structure analysis from far-ultraviolet (UV) protein CD spectra and to the fingerprint information that can be obtained from UV-visible (near-UV) CD spectra. Subsequently, the use of CD spectroscopy and of stopped-flow CD as an important tool to elucidate folding and unfolding of proteins is described. An

updated survey of CD applied to proteins at interfaces is given in a separate section. Finally, the chapter closes with a summary of instrumental aspects of circular dichroism.

II. BASIC THEORY

A. Introduction to Optical Spectroscopy

Light is a rapidly oscillating electromagnetic field. The interaction of electromagnetic waves with matter can be used to extract information about biological macromolecules. This is the basis of spectroscopy. Optical spectroscopy is concerned with wavelengths between about 150 nm (vacuum UV) and 6000 nm (far infrared (IR)). Optical spectroscopy involves irradiation of the sample with some form of electromagnetic radiation, followed by measurement of a spectral property such as scattering, absorption, or emission and, finally, by interpretation of the measured spectral parameters to obtain specific molecular information. Electromagnetic radiation has two waves at right angles to each other; one is electric (E) and the other is magnetic (H) (see Fig. 1). They are produced by oscillating electric or magnetic dipoles and are propagated through vacuum with the velocity of light \( c = 3 \times 10^8 \text{ m s}^{-1} \). In Fig. 1 plane or linearly polarized light is shown, because the E vector oscillates in one plane. Unpolarized light contains oscillations of the E wave in all directions perpendicular to the direction of propagation (P). The frequency (\( \nu \)) and wavelength (\( \lambda \)) of a wave are related by the equation

\[
\nu = \frac{c}{\lambda}
\]

(1)

Frequency can be directly converted into energy units using the relationship

\[
E = h\nu
\]

(2)

where \( h \) is Planck's constant \( (h = 6.63 \times 10^{-34} \text{ J s}) \). Units of energy (J mol\(^{-1}\)), frequency (Hz), and wavelength (m) are all used in optical spectroscopy. Also used is the wavenumber (\( \nu' \)), defined as the inverse of the wavelength in centimeters:

\[
\nu' = \frac{1}{\lambda} = \nu/c
\]

(3)

The wave number is thus the number of waves per centimeter.

B. The Phenomenon of Optical Activity: Circularly Polarized Light

A molecule is optically active if it interacts differently with left- and right-handed circularly polarized light. Such interaction can be detected in two ways: either as a differential change in velocity of the two beams through the sample (optical rotatory dispersion) or as differential absorption of each beam (circular dichroism). These phenomena are usually measured in the wavelength range 180–700 nm connected with electronic transitions. Optical activity measurements applied to biological macromolecules provide information on molecular conformation, conformational changes upon ligand binding, and secondary structure. Circular dichroism is more frequently
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Fig. 1 Plane-polarized light.

used than optical rotatory dispersion (ORD). ORD will therefore not be treated in this chapter. To observe CD, a molecule must satisfy two prerequisites: it must have chirality (as in L-amino acids) and be chromophoric (the molecule must absorb light of certain wavelength). In biological macromolecules chirality is often induced by asymmetrically placed neighboring groups.

Circularly polarized light is shown in Fig. 2a. If one follows the point of the E vector, the movement is like a helical path around the axis of propagation (P). This motion can be left- or right-handed depending on the sense of rotation with respect to the direction of propagation. Plane-polarized light can be composed of a sum of left- and right-handed circularly polarized waves of equal amplitude (see Fig. 2b). When an optically active compound preferentially absorbs one of the circular components, the outgoing beam is elliptically polarized (Fig. 2c). This ellipticity is determined by the axial ratio of the ellipse. CD can be expressed in molar ellipticity [θ] (see later).

It is equally easy to represent a circularly polarized wave as the sum of two plane-polarized waves, 90° out of phase and with their electric vectors perpendicular (see Fig. 2d).
Fig. 2  (a) Circularly polarized light. Only the electric field is shown. (b) Plane-polarized light as the sum of two circularly polarized waves of equal amplitude. (c) Elliptically polarized light upon leaving an optically active, light-absorbing compound. (d) Circularly polarized wave represented as sum of two plane-polarized waves, shifted 90° out of phase and with their electric vectors perpendicular.
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C. Circular Dichroism: The Molecular Basis of Optical Activity

CD at a given wavelength is defined as

$$\Delta \epsilon = \epsilon_L - \epsilon_R$$  \hspace{1cm} (4)

$\Delta \epsilon$, expressed in (mol dm$^{-3}$)$^{-1}$ cm$^{-1}$, is much smaller (approximately three to five orders of magnitude) than the molar extinction coefficient $\epsilon$ itself. The relation between molar ellipticity $[\theta]$, expressed in degree cm$^2$ dmol$^{-1}$, and $\Delta \epsilon$ is (the derivation can be found in Ref. 1 or 2):

$$[\theta] = 3300 \Delta \epsilon$$  \hspace{1cm} (5)

How can one obtain a molecular picture of CD? We know that when a molecule is excited with light, a displacement of charge takes place. This linear displacement is associated with an electric transition dipole moment, denoted by $\mu$. The transition can also have a circular component, which in turn can generate a magnetic dipole moment $m$ perpendicular to the circular plane (Fig. 3). Optical activity requires that circularly polarized light induces both dipole moments in more or less the same direction in the molecule. As the dipole strength is a measure of the intensity of an optical absorption transition, the rotational strength is the determining factor in CD. The rotational strength ($R$) is equal to the integrated dichroism over the absorption band:

$$R = \frac{3hc}{8\pi^2 N_0} \int \frac{\Delta \epsilon}{\lambda} d\lambda$$  \hspace{1cm} (6)

($N_0$ is Avogadro’s number). The rational strength can be computed from quantum

Fig. 3 Schematics of light-induced charge displacement in a molecule. (a) Pure electronic absorption: $\langle \Psi_0 | \mu | \Psi_0 \rangle$. (b) Pure magnetic absorption: $\langle \Psi_0 | m | \Psi_0 \rangle$. (c) Optical activity: $\langle \Psi_0 | \mu | \Psi_0 \rangle \otimes \langle \Psi_0 | m | \Psi_0 \rangle$. 

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\[\text{(Image of diagrams is not transcribed)}\]
mechanical principles and from knowledge of the wave functions describing ground ($\Psi_0$) and excited states ($\Psi_e$). The result is

$$R = \text{Im}([\Psi_e|\mu|\Psi_e][\Psi_e|m|\Psi_e])$$  \hspace{1cm} (7)

The vector $\mu$ and $m$ are now the electric dipole operator and magnetic dipole operator, respectively, because they operate on the wave functions. "Im" means the imaginary part of the expression that follows in Eq. (7). For each electron, $m$ can be written as the following cross-product:

$$m = \frac{e}{2mc} (r \times p)$$  \hspace{1cm} (8)

where $e$ and $m$ are the charge and mass of the electron, $p$ is the momentum operator, and $r$ is the position operator of the electron. The cross-product $r \times p$ is the orbital angular momentum of an electron. Therefore the magnetic transition dipole [right-hand part of right-hand side of Eq. (7)] corresponds to a circulation of charge of current loop (see Fig. 3). The dipole strength is then the square of the light-induced electric dipole (see Fig. 3).

Optical activity involves both electric and magnetic interactions. Equation (7) implies that $R$ is a dot product and thus a scalar (a so-called physical observable, just a number). We can express this as follows:

$$R = |\mu||m| \cos \delta$$  \hspace{1cm} (9)

where $|\mu|$ and $|m|$ are the magnitudes of the vectors and $\delta$ is the angle between the two dipole moments. For a molecule to be optically active the magnetic dipole operator $m$ must have a component parallel to the electric dipole operator $\mu$. To accomplish this the molecule must be asymmetric. Many helical macromolecules have intense optical activity, because the helical structure facilitates a helical flow of charge.

Figure 4a shows a circularly polarized wave that interacts with a right-handed helical fragment. The circularly polarized light wave is represented by two plane-polarized components 90° out of phase. The electric field component $E_1$ will induce an oscillating electric dipole moment $\mu$ with a component parallel to the helix axis. Similarly, an oscillating magnetic field component $H_2$ will induce a current parallel to the helix axis. According to Maxwell's laws, this depends on $dH_2/dt$ (first derivative with time). $dH_2/dt$ is in phase with $E_1$ and both contribute synchronously to electron displacement on the helical path or, in other words, create an oscillating electrical dipole moment $\mu$ and magnetic dipole moment $m$ parallel to the helix axis. According to Eq. (9) this corresponds to maximal rotational strength because $\delta = 0°$. The components $E_2$ and $H_1$ do not contribute because they do not result in electron displacement.

One can now intuitively understand why a molecule such as coronene is optically inactive and why a molecule such as hexahelicene has intense optical activity (Fig. 4c). Coronene is symmetric ($D_{4h}$ symmetry). Excitation with circularly polarized light of the proper energy induces a charge displacement of $\pi^*$ electrons on a circular path ($\mu$). From Maxwell's laws of electromagnetism we know that a magnetic moment
Fig. 4  (a) The interaction of circularly polarized light with a helical molecule. (b) Coronene and hexahelicene.

m will be induced that is perpendicular to the plane in which the electrons flow. The angle $\delta$ is $90^\circ$ [see Eq. (9)] and $R = 0$. Hexahelicene is strongly optically active because the molecule adopts a helical configuration.
D. Exciton Splitting Illustrated with a Dimeric Molecule

When one makes, e.g., a 10 μM solution of reduced nicotinamide adenine dinucleotide (NADH) and subsequently measures its absorbance at 340 nm to determine its exact concentration, we rely on the assumption that the molecules do not interact with each other and on the extinction coefficient being the same for all molecules. We can safely assume this because, when the average distance between the molecules in calculated, it turns out that they are completely isolated and do not interact. In biological macromolecules, however, we are dealing with molecular assemblies. Examples are proteins in which peptide chromophores are linked together; nucleic acids, which are polymers of deoxyribonucleotide ribonucleotide units forming single or double-helical strands; and light-harvesting proteins in which many chlorophyll molecules are dispersed. In all these cases the chromophores are feeling each other's presence; they are definitely interacting. All biological aromatic molecules in the ground state have some permanent dipole moment (a few Debye). When these molecules are brought to the excited state by absorption of light of the proper energy, it can be generalized that these dipole moments become much larger (in some cases even 5 to 10 times). A molecule in the excited state has a different charge distribution and is much more polar than in the ground state. Consequently, the dipolar interactions are more pronounced in the excited state and strongly influence the absorption spectrum.

These effects are illustrated using two identical chromophores that are covalently linked together (see Fig. 5a). The two relative orientations of the dipoles in the two molecules correspond to a situation in which the dipoles oscillate in phase (plus state, left) or out of phase (minus state, right). Because of dipolar interaction an absorption spectrum is split into two bands corresponding with two transitions \( \nu_+ \) and \( \nu_- \). (Fig. 5b). This is called exciton splitting to emphasize the fact that the excited state is affected rather than the ground state. The transition probabilities (or dipole strength; see earlier) for the two states are given by

\[
D_\pm = D_0(1 \pm \cos \alpha) \tag{10}
\]

in which \( \alpha \) is the angle between the transition dipoles \( \mu_1 \) and \( \mu_2 \) (see Fig. 5c). Note further that \( D_+ + D_- = 2D_0 \) for each angle \( \alpha \). The frequencies of the new absorption bands are

\[
\nu_\pm \equiv \nu_0 \pm \nu_{12} \tag{11}
\]

where \( \nu_{12} \) is a measure of the dipole-dipole interaction between \( \mu_1 \) and \( \mu_2 \).

\[
\nu_{12} = D_0 \left(\frac{\cos \alpha - 3 \cos \gamma \cos \beta}{\rho^3}\right) \tag{12}
\]

The dominant contribution to the rotational strengths \( R_+ \) and \( R_- \) of the two states arising from dipolar interaction is given by (see Ref. 2 for derivation)

\[
R_\pm = \pm \left(\frac{\pi}{2\lambda}\right) r_{12} \cos \delta (\mu_1 \times \mu_2) \tag{13}
\]

in which \( \mu_1 \times \mu_2 \) is a cross-product and \( \delta \) is the angle between \( r_{12} \) (Fig. 5c) and the normal to the plane formed by \( \mu_1 \) and \( \mu_2 \). The product is a measure of helical twist in going from \( \mu_1 \) to \( \mu_2 \).
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Fig. 5  (a) Orientation of transition dipole moments in a dimer. (b) Exciton splitting of an absorption spectrum into two bands by interacting transition dipoles. The CD spectrum shows a positive Cotton effect for the $v_+$ transition and a negative one for the $v_-$ transition. (c) The interaction between two dipoles $\mu_1$ and $\mu_2$ can be described by the angles $\alpha$, $\beta$, and $\gamma$ and the position vector $r_{12}$. 
III. INFORMATION FROM CIRCULAR DICHROISM SPECTRA OF PROTEINS

A. Circular Dichroism of Proteins

All amino acids except glycine are asymmetric and hence optically active. Apart from aromatic amino acids, the chromophoric group in proteins is the peptide bond. The arrangement of these bonds in organized structures such as α-helix, β-sheets (parallel and antiparallel), and β-turns is the reason that such distinct and different CD spectra are observed between different proteins. Figure 6a shows an example of the CD spectrum of a synthetic polypeptide in α-helix conformation (adapted from Ref. 6). For comparison the absorption spectrum is also given.

Three electronic transitions can be observed:

1. At about 210–220 nm a weak absorption band can be seen in the absorption spectrum that has considerable optical activity in the case of an α-helix. This is the $n,π^*$ transition, in which the nonbonding electrons of the carbonyl oxygen are promoted to the antibonding π orbital. The $n,π^*$ transition has a low extinction coefficient (is forbidden) but high rotational strength. The latter can be visualized as a charge rotation about the C=O bond accompanied by the generation of a magnetic moment (Fig. 6b).

2. In the absorption spectrum two shoulders are seen at 185 and 205 nm. These correspond to a single allowed $π,π^*$ transition, which has a high extinction coefficient. The π-electrons are delocalized over the whole peptide fragment. In an α-helix the peptide chromophoric groups feel each other’s presence and due to this interaction the $π,π^*$ transition is split into two bands (positive and negative) with the sign change occurring at about 200 nm. This is a clear example of exciton splitting.

3. The spectrum below 180 nm is less well understood. There is probably another $ν,π^*$ transition at 160 nm. This spectral region (and lower) is called the vacuum UV and the optical energies are such that the localized σ-electrons can be excited into the antibonding σ-orbital ($σ,σ^*$ transition).

B. Prediction of Protein Secondary Structure

Figure 6a shows the CD spectrum of a polypeptide in the α-helix conformation. Changing the orientation of the peptide bond into other organized or nonorganized structures has a drastic influence on the CD spectrum as illustrated for poly-L-lysine in Fig. 7a (adapted from Ref. 7).

In random coil conformation the $n,π^*$ transition does not exhibit dichroism, and the exciton splitting of the $π,π^*$ transition is completely absent. The β-sheet conformation shows a CD spectrum intermediate between the other two spectra. It has been pointed out by Johnson (8) that one should record CD spectra to about 180 nm in order to deduce spectral features that are characteristic for various conformations. In this way the information content of CD is increased and computer predictions can be made with higher confidence. For instance, it is possible to make distinctions between antiparallel or parallel β-sheets and a β-turn.

Nonetheless, in the 1970s the spectra shown in Fig. 7a served as a basis set for protein secondary structure prediction. As an approximation the protein is built up of a linear combination of α-helical, β-sheet, and random coil structures. A
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Fig. 6 (a) CD and absorption spectra of poly-γ-benzyl-L-glutamate in α-helix conformation adapted from Ref. 6. (b) n- and π*-orbitals of the carbonyl peptide bond. The magnetic moment is due to charge rotation about the C–O bond.
Fig. 7  (a) CD spectra of poly-l-lysine in various conformations (adapted from Ref. 7). (b) CD spectra for α-helix, β-sheet, and random coil conformations recovered from CD spectra of proteins of known 3D structure (adapted from Ref. 9).
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Multicomponent analysis is then made to match observed and calculated CD spectra according to the following linear equations for different λ:

\[ x_{\text{obs}}(\lambda) = \alpha x_{\alpha}(\lambda) + \beta x_{\beta}(\lambda) + \gamma x_{\gamma}(\lambda) \]  

(14)

where \( x_{\text{obs}} \) is the observed CD spectrum at wavelength \( \lambda \) of the protein of interest. The \( \chi \) values at the right side of the equation are the spectra of the basis set, and \( \alpha, \beta, \gamma \) are the fractions to be determined by solving simultaneously the set of linear equations given by Eq. (14) (of course, constraints may be applied such as \( \alpha + \beta + \gamma = 1 \) and \( \alpha, \beta, \gamma > 0 \)). One should, however, be cautious in using this approach. Proteins do not contain large extended helices and sheets like those of the poly-amino acid model systems. In addition, helices in proteins can be densely packed and helix-helix interactions affect the CD spectra (the same applies for sheet-sheet interactions). Finally, as indicated earlier (8), several types of sheet conformations and turns are found in proteins. These aspects were noted in the beginning of the 1970s. Consequently, rather than using polypeptides, a set of "known" proteins is now chosen to compute the basis spectra. Known proteins are proteins whose three-dimensional (3D) structure has been determined using X-ray diffraction and whose fractions \( \alpha, \beta, \gamma \) can thus be evaluated. By solving a set of equations corresponding to Eq. (14), a CD spectral basis set can be recovered. A typical example of such a basis set is presented in Fig. 7b (adapted from Ref. 9).

A comprehensive review appeared (10) that compares the various methods of obtaining structural information from CD data. The computational methods, developed during the past two decades, include singular value decomposition, ridge regression, variable selection, self-consistent method, and neural networks. Because we have used the ridge regression method (CONTIN) in analyzing CD spectra, this method will be briefly outlined (details can be found in Ref. 11). The CD spectrum of an unknown protein is directly fitted to a linear combination of spectra from a large database of proteins with known conformations. CONTIN is a variation of the method of least-squares minimization. In this method, the contribution of each reference spectrum is kept small unless it contributes significantly in obtaining the optimal fit. Use is made of a so-called regularizer, which eliminates any biased contribution of a particular protein in the reference set. In this way the least-squares solution is stabilized, bearing in mind that many other solutions could be equally valid in multiparameter fitting.

An example of CD analysis to retrieve the secondary structure content of the flavoprotein lipooamide dehydrogenase (from Azotobacter vinelandii) using CONTIN is given in Fig. 8 (12). The experimental and calculated CD spectra and the residuals between both spectra are shown. The \( \alpha \)-helix content determined (30%) is in excellent agreement with that from the X-ray structure. The \( \beta \)-sheet and \( \beta \)-turn contributions are somewhat higher than inferred from the 3D structure. The latter is due to the fact that the C-terminal domain of this protein is not well defined in the 3D structure. C-terminal deletion mutants of lipooamide dehydrogenase have the same secondary structure content as the X-ray structure (12).

C. Induced Circular Dichroism in Proteins: Fingerprint Function

CD of an optically active residue in a protein is very sensitive to its microenvironment. Therefore the technique can be exploited to detect conformational changes in proteins
Fig. 8 Experimental and fitted far-UV CD spectra of *Azotobacter vinelandii* lipoamide dehydrogenase. The residuals between experimental and fitted curve (top) are randomly distributed around the zero line and indicate optimal fitting. (Redrawn from Ref. 12)

resulting from ligand (substrate, inhibitor, effector) binding, variation in temperature, change in pH, addition of denaturant, etc. The aromatic amino acids tryptophan, tyrosine, and phenylalanine are examples of chiral, light-absorbing molecules that yield very distinct CD spectra in proteins. Other examples are the flavin prosthetic group in flavoproteins, heme in heme proteins, and pyridoxal phosphate and NADH in particular enzymes. All these molecules have in common that the chromophoric group is placed in an asymmetric protein environment. Chirality is then induced by the
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protein. In such a complex protein environment it is very difficult to derive the molecular origin of the optical activity. However, one can use CD as a convenient tool to monitor conformational changes and to establish thermodynamic information associated with these changes. A good review of the use of CD in conjunction with light absorption and fluorescence to investigate protein unfolding is given in Ref. 13.

In principle, disulfides in proteins also have some chirality. The optical activity arises from the electronic transition (near 250 nm), being dependent on the \( -S\text{--}S\text{-}dihedral\) angle.

As an example of the fingerprint function of CD, the CD spectra of flavin adenine dinucleotide (FAD) bound to wild-type lipoamide dehydrogenase and to a deletion mutant are shown in Fig. 9 (12). Free FAD exhibits very weak CD (not shown), and bound FAD shows high optical activity in the near-UV band. Deletion of 14 C-terminal residues of the protein changes the microenvironment of the flavin, which is reflected by a drastically changed CD spectrum. The CD spectrum of the deletion mutant resembles that of a reductase such as ferredoxin-NADPH oxidoreductase. Each flavoprotein with particular function has its own characteristic

![Graph](image_url)

**Fig. 9** Visible and near-UV CD spectra of *A. vinelandii* lipoamide dehydrogenase and a deletion mutant. Solid line, wild type; dashed line, deletion mutant. (Redrawn from Ref. 12.)
CD spectrum, whereas the light absorption spectra are much less distinctive. CD thus clearly has a fingerprint function.

D. CD Spectroscopy and the Study of Protein (Un)Folding

As described in the previous sections, CD spectroscopy provides information about the secondary (far-UV CD) and tertiary (near-UV CD) structure of a protein. The technique is therefore routinely applied to characterize the native state of a protein molecule. Estimates of the amounts of the various secondary structure elements (i.e., α-helices, β-strands, and coil regions) can be obtained using this technique. In the native state of a protein, these secondary structure elements are ordered in a highly specific manner. The arrangement of these elements and the specific conformations of the side chains of the amino acid residues determine the three-dimensional structure of a protein. Formation of the complicated three-dimensional protein structures is essential for proteins to be biologically active. The functional properties of proteins depend on these specific structures (14).

Since the first three-dimensional structures of proteins were elucidated, the problem of how proteins fold to such complicated tertiary and quaternary structures has been debated. It was Anfinsen with his coworkers (15,16) who demonstrated that proteins can fold unassisted and reversibly to their native three-dimensional state. Consequently, all the information required to define the tertiary fold is encoded in the amino acid sequence (17). It was readily realized that protein folding does not take place via a random sampling of conformational space. Even for small proteins, such as flavodoxin from *A. vinelandii* (179 residues), simple calculations show that this process would take longer than the lifetime of the universe (18). Protein folding, however, usually takes place on the millisecond to minute time scale. To explain the latter, the concept of protein folding pathways arose. A protein folds along a protein folding pathway from the fully unfolded to the fully folded native state. For most proteins under physiological conditions, the native three-dimensional structure is the thermodynamically most favorable state, which thus predominates.

The understanding of how proteins fold should answer a few related questions: what is the physical basis of the stability of the folded protein conformation; what processes determine that a protein adopts its native conformation; what are the rules that link the amino acid sequence with the three-dimensional structure of a protein; and finally, can the three-dimensional structure of a protein be predicted from its amino acid sequence (19–21)? Besides the fact that the answers to these questions are of academic interest, knowledge about protein folding is now being exploited in many practical applications in biotechnology and thus is also of industrial importance (22). General rules governing protein folding are now beginning to emerge (23,24). A "new view" on protein folding replaces the idea of distinct folding intermediates on a specific folding pathway with the idea of an ensemble of protein conformations that fold via parallel multipathway diffusion-like processes (25).

CD spectroscopy plays an important role in the study of protein folding as it allows the characterization of secondary and tertiary structures of proteins in native, unfolded, and partially folded states. As described earlier, the CD spectrum of a native protein reflects its three-dimensional structure. As a typical example, the far-UV CD spectrum of native *A. vinelandii* apoflavodoxin is shown in Fig. 10. Under physiological conditions apoflavodoxin adopts the α/β doubly wound topology, which consists of a
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![Graph showing CD spectra of native and denatured forms of C69A apoflavodoxin from A. vinelandii and of apoflavodoxin in 4M guanidinium hydrochloride. The spectra were recorded at 25°C in 100 mM potassium pyrophosphate, pH 6.0. (Adapted from Ref. 113)]

Fig. 10  Far-UV CD spectrum of native C69A apoflavodoxin from A. vinelandii and of apoflavodoxin in 4M guanidinium hydrochloride, respectively. The protein concentration was 4 μM and the spectra were recorded at 25°C in 100 mM potassium pyrophosphate, pH 6.0. (Adapted from Ref. 113)

denatured

Native

Wavelength (nm)

$\left[\theta\right] \times 10^{3}$ (deg cm$^2$ dmol$^{-1}$)

five-stranded parallel β-sheet surrounded by α-helices at either side of the sheet (25a), as is also observed for holoflavodoxin (26). The presence of a sufficient amount of denaturant usually causes a protein to unfold. The CD spectrum of apoflavodoxin in 4 M guanidinium hydrochloride (Fig. 10) is typical for unfolded proteins exhibiting random coil behavior.

A combination of circular dichroism and fluorescence spectroscopy is often used to demonstrate whether equilibrium (un)folding of proteins takes place via a two-state mechanism or whether it involves protein folding intermediates (13). In case of apoflavodoxin, the combination of both techniques clearly demonstrates the presence of a relatively stable folding intermediate in the denaturant-induced equilibrium (un)folding of the protein (Fig. 11). The intermediate is characterized by loss of native tertiary interactions as its fluorescence emission intensity is decreased compared with that of the native state. The intermediate, however, has an appreciable amount of secondary structure as inferred from CD spectroscopy. Folding intermediates with such characteristics have been observed for a few globular proteins and are called molten globules (27). The occurrence of a molten globule–like folding intermediate is also observed during the thermal unfolding of apoflavodoxin: CD and fluorescence spectroscopy give drastically different thermal midpoints of unfolding (Fig. 12). Instead of using fluorescence spectroscopy, near-UV CD spectroscopy can be used as well to detect the immobilization of aromatic residues in the tertiary structure of a protein during folding. The molten globule state of α-lactalbumin, which is induced by
lowering the pH of the solution, is unfolded as inferred from near-UV CD but is
native-like on basis of far-UV CD (28).

Stopped-flow CD studies have become increasingly popular in the kinetic anal-
ysis of protein unfolding and refolding. Modern CD instruments are useful for rapid
reaction measurements as the lower limit of their time resolution, estimated to be
as short as 0.2 ms, is short enough compared with the mixing dead time of the
stopped-flow method (29). This mixing time is usually a few to ten milliseconds.
The stopped-flow CD technique is widely used in studies of the kinetics of structural
transitions of proteins and other biomolecules. Application of the stopped-flow
CD technique to studies of protein folding is, however, rather new (29). The technique
is effective in detecting and quantitating the secondary structure formed in the tran-
sient intermediates during protein folding (29).

If the denaturant-induced unfolding of a protein is reversible, as is the case for,
e.g., the apoflavodoxin of A. vinelandii mentioned before, the refolding of an unfolded
protein can be studied by diluting the denaturant. Stopped-flow CD spectroscopy then
provides an indication of the overall extent of structural organization in the refolding
molecules, in terms of both secondary structure (from far-UV CD) and tertiary struc-
ture (from near-UV CD) (17). To initiate protein refolding a concentration jump of the
denaturant is created and special care is required to perform the stopped-flow
Fig. 12. Temperature-induced equilibrium unfolding of 2 μM C69A apoα-lactoadoxin from A. vinelandii as monitored by fluorescence emission at 350 nm (■) and by CD at 222 nm (○). The fluorescence excitation wavelength was 280 nm. The spectra were recorded in 100 mM potassium pyrophosphate, pH 6.0. (Adapted from Ref. 113)

Experiments. The leap to a native condition from the unfolded state requires a wide concentration jump, and a mixing apparatus with a high dilution ratio (10:1 or even more) is thus necessary (29). Mixing apparatus with two or more driving syringes have been designed in which plungers are driven by either stepping motors or pneumatics and control the solution delivery. In typical protein folding-unfolding experiments, a heavy solution of urea or guanidinium hydrochloride is mixed with pure aqueous buffer. The efficient mixing of solutions of different densities is a formidable challenge for a stopped-flow instrument. Various manufacturers have tackled this differently. Currently, several commercial stopped-flow instruments combined with CD spectrometers are available. Manufacturers are, among others, Biologic (France), Jasco (Japan), Aviv (USA) and Applied Photophysics (UK).

The information obtained so far by stopped-flow CD spectroscopy has been limited by the time and structural resolution of the technique. The time resolution is currently on the millisecond scale, which does not permit the study of early folding events, although further developments are expected (30). It is generally observed by CD spectroscopy that a large complement of secondary structure is formed within the first few milliseconds of protein folding (Fig. 13). This process takes place within the dead time of the measurement using the current stopped-flow techniques. As an example, Fig. 13 shows kinetic refolding curves of β-lactoglobulin measured by the ellipticities at 219 (far UV) and 293 nm (near UV) and a kinetic unfolding curve of the same protein measured by the ellipticity at 220 nm (31). Similar curves were
Fig. 13  Kinetic refolding (a, b) and unfolding (c) curves of bovine $\beta$-lactoglobulin A measured by the stopped flow CD at pH 3.2 and 4.5°C. The refolding was induced by a concentration jump on guanidinium hydrochloride from 4 to 0.4 M and measured at 293 nm (a) and 219 nm (b). The unfolding was induced by a concentration jump of guanidinium hydrochloride from 0 to 4 M and measured at 220 nm (c). $\theta_N$ and $\theta_U$ denote equilibrium CD values in the native (N) and unfolded (U) states, respectively. (Redrawn from Ref. 29)
obtained by other groups who used the stopped-flow technique to study the refolding of small globular proteins from their unfolded state in either urea or guanidinium hydrochloride (e.g., 30,32–41). In the aromatic region, however, the changes in the CD spectra from the unfolded to the folded state (Fig. 13) can often be observed kinetically during refolding of proteins (29). The transient accumulation of an intermediate state I that contains secondary structure elements but is unfolded in terms of the aromatic CD spectra is observed for some proteins (27).

Characterization of the I state by CD spectroscopy is made possible by measurements of the kinetic progress curves of refolding at various wavelengths. Because the formation of the I state in the burst phase occurs much faster than the subsequent folding events, the wavelength dependence of the CD value obtained by extrapolation to the zero time of the observed refolding curve corresponds to the I state (29). It was demonstrated in this manner that the CD spectra of the I state for lysozyme and α-lactalbumin are similar to each other and to the equilibrium CD spectra of the molten globule state of α-lactalbumin. This suggests that the folding of both proteins takes place via the initial collapse of the polypeptide chain to a molten globule state. Subsequent relatively slow rearrangements of the protein structure lead to the fully folded native state of the protein.

CD spectroscopy provides average information about the secondary and tertiary structure of protein folding intermediates. However, detailed knowledge of the partially structured states of such folding intermediates is essential for understanding protein folding. CD spectroscopy cannot give any specific information about where the secondary structure such as an α-helix or a β-sheet is formed within a molecule (29). It has become possible, due to drastic improvements made in multidimensional nuclear magnetic resonance (NMR) spectroscopy, to characterize such intermediates using NMR (37,42–45). Use is made of differences in exchange rate of individual amide protons versus deuterium oxide in the folding intermediates. It is not necessary to study the folding intermediates themselves, as they exist only transiently on the time scale of milliseconds to seconds due to the high cooperativity of protein folding. Amide proton exchange pulse labeling against deuterium oxide (using rapid quench techniques) of the amide protons in the folding intermediates makes it possible to characterize the conformations of the intermediates using the two-dimensional NMR spectra of the native state. Pioneering initial structural characterizations of folding intermediates using this method have been obtained for a few proteins, including cytochrome c, ribonuclease A, guinea pig α-lactalbumine, and hen lysozyme (37,42–44). The most detailed structural characterization, however, has until now been possible only for stable analogues of the folding intermediates of bovine pancreatic trypsin inhibitor (BPTI) (46–51).

Stopped-flow CD data on protein folding are now used to complement results obtained via stopped-flow fluorescence and mass spectrometry experiments. The stopped-flow CD technique is of particular value in conjunction with the pulselabeling experiments. In the case of lysozyme, experiments in the far UV indicate that a large complement of secondary structure is formed within the first few milliseconds of folding as observed for several other proteins. The rate of secondary structure formation, however, is actually much higher than the rate at which exchange protection develops, suggesting that amide proton protection probably arises from stabilization of already formed helical structure in lysozyme (17).
In conclusion, both CD and stopped-flow CD are very valuable techniques for studying protein folding. They have significantly contributed in our understanding of how proteins fold. In combination with results from complementary techniques, both CD and stopped-flow CD are expected to play an important role during the coming years in the deciphering of the protein folding problem.

IV. CIRCULAR DICHOISM OF PROTEINS AT INTERFACES

In this section we describe CD studies of proteins located at membranes, solid carrier–air and solid carrier–water interfaces. The preparation of the samples and the influence of the system on the CD spectral characteristics is discussed. Some examples of reported CD studies will be presented to illustrate the strength of this technique to gain information on the structural properties of proteins at interfaces.

A. Proteins in Membranes or Membrane-Mimicking Systems

When protein-lipid complexes are the subject of a CD study, it is inevitable that light scattering caused by the relatively large particles (>wavelength) can interfere with the interpretation of the data (52). The consequence of light scattering is primarily a strong reduction of the light intensity as sensed by the detection system, resulting in a lower signal-to-noise ratio. However, if the two rotations of light are scattered differently, a CD artifact can be created that could hinder the interpretation of protein CD. The presence of CD artifacts as a result of particle scattering can generally be identified by the appearance of CD outside the normal protein absorption bands.

The extent of differential light scattering can be minimized by sonication of the membrane particles, resulting in small unilamellar vesicles (SUVs) (53). It should be noted, however, that the stability of these small vesicles is low and spontaneous fusion to larger structures occurs in time. A more reproducible method for studying proteins in membrane systems is obtained by extrusion techniques, yielding large unilamellar vesicles (LUVs). The pore size of the polycarbonate filters used determines the size and homogeneity of the membrane structures (54). Due to the relatively high turbidity, the applicability of multilamellar vesicles, obtained by resuspension of lipid films in aqueous buffer followed by several heating-cooling cycles, for CD studies is less favorable. Final lipid concentrations of 5–10 mM SUVs or LUVs are feasible (using 0.01–0.02 cm cuvette path lengths) for reliable CD measurements of proteins in these systems (55,56), allowing systems with lipid-to-protein ratios higher than 100:150 to be studied by CD.

Micellar systems are an alternative widely used for CD studies. They mimic membrane environments and usually eliminate the problem of differential light scattering. In the literature a large variety of types of detergents have been reported. In some cases lysophospholipids have been used (57,58), but sodium dodecyl sulfate (SDS), a negatively charged surfactant, is the one most frequently used. Because the protein conformation might be strongly dependent on the membrane surface charges, neutral [for example, dodecylmaltoside, dimethyl dodecylamine-N-oxide (LDAO), octyl-glucoside, or Triton] or zwitterionic (e.g., dodecylphosphocholine) detergents have been used extensively as well. Both can be purchased or synthesized in an easy manner (59,60).
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Besides light scattering due to particles, differential absorption flattening can also occur in parts of the far-UV CD spectra of membrane-embedded proteins (61–63). This spectral artifact is related to the deviation from an ideal homogeneous distribution of chromophores in the solution that is necessary to fulfill the criteria for Beer’s law. When the chromophores are densely packed the total cross-sectional area is smaller than when the particles are uniformly dispersed. Consequently, the extent of flattening depends on both the size of the particles and the number of chromophores present in each particle. Especially in spectral regions where protein absorbance is strong, absorption flattening can contribute seriously. Hence, the extent of absorption flattening is wavelength dependent. Some studies have tried to determine the “flattening” factor for various wavelengths (53), but because these factors are system dependent a straightforward more general procedure to correct CD spectra for flattening effects cannot be given. A way to identify the presence of absorption flattening is by sonication of the membrane fragments as demonstrated by Mao and Wallace (53). They showed that disruption of purple membrane fragments into smaller particles reduced their size and the number of chromophores per particle. For this reason, detergent-solubilized membranes can be considered free of absorption flattening. All studies in which absorption flattening was apparent dealt with integral membrane proteins. In such systems the proteins are two-dimensionally concentrated in a membrane and often have a strong interaction with each other, forming, for example, defined hexagonal arrangements in the membrane, a case encountered in bacteriorhodopsin. However, it should be noted that absorption flattening effects can also occur in protein films or even in solutions containing densely packed proteins.

Because all protein absorption bands observed with far-UV CD reflect \( \pi, \pi^* \) and \( \alpha, \pi^* \) transitions, it is obvious that the intensity and frequency position of these bands depend on the dielectric constant of the local environment. Comparable to the CONTIN method described earlier in this chapter, an effort has been made to establish reference spectra for integral membrane proteins based on the CD spectra of 30 different detergent-solubilized integral membrane proteins with known secondary structure content (64). From this analysis it was shown that two reference spectra are needed to describe the helical content of an integral membrane protein: one assigned to \( \alpha \)-helices in soluble domains and the other accounting for transmembrane helices. In this latter spectrum the positive CD band below 200 nm (\( \pi, \pi^* \) transition) was red shifted approximately 5 nm, whereas the negative band at 222 nm (\( \pi, \pi^* \) transition) was slightly more negative than that at 208 nm (\( \pi, \pi^* \) transition). In addition, the rotational strength was greater for the transmembrane helices. The latter could be attributed to (a) the fact that helices spanning a membrane are generally two times larger than helices in soluble domains and (b) the lower dielectric constant.

1. Integral Membrane Proteins

In the literature only CD spectra of integral membrane proteins in detergent-reconstituted systems can be found. A few exceptions exist, with the CD spectrum of bacteriorhodopsin (BR) in purple membranes of *Halobacterium halobium* as a typical example (53). However, because of absorption flattening the secondary structure of BR determined by CD deviated strongly from the reported X-ray structure. The spectral distortion was reduced only slightly by sonication of the membranes. However, when changes in CD are monitored, as, for example, reported for the dependence of the kinetics of folding of BR in vitro on pH or membrane composition (65), the spectral
distortions do not affect the results. Reconstitution of BR in dimyristoylphosphatidylcholine (DMPC) membranes and subsequent extensive sonication of the sample resulted in CD spectra that revealed after analysis a secondary structure content comparable to the X-ray structure. Whereas BR is known to consist of seven transmembrane helices, almost no detailed information is available on β-structured integral membrane proteins. Only one study reported CD spectra of the voltage-gated channel VDAC (or mitochondrial porin) isolated from Neurospora crassa reconstituted in model membranes (66). Indeed, a large content of β-structure was found, in agreement with structure predictions and neutron diffraction data.

More quantitative CD analysis of integral membrane proteins has been carried out in micellar systems (for an extensive overview, see Ref. 64). An example is diacylglycerol kinase, a relatively small integral membrane protein (13 kDa) that spans the membrane three times. This protein is still active when reconstituted in particular mixed micellar systems. By variation of the molar ratio of β-maltoside and SDS this protein could be unfolded reversibly as monitored by CD, and the stabilities of the cytoplasmic domain and the membrane-embedded part could be established (67).

The treatment of membranes with detergents in order to break up the particles or to extract the integral membrane proteins should always be performed with great care. On treating purple membranes of H. halobium with Triton X-100, it was found that particular membrane lipids were removed. As a result, the kinetics of the photocycles of this proton pump were significantly altered (68). Both near- and far-UV CD analysis showed that this treatment affected both the secondary structure of BR and its ability to trimerize. This could be reversed by reintroduction of an extract of membrane lipids to the protein. Alternatively, the relatively small dimensions of micelles could prohibit such a solubilized integral membrane protein attaining the proper structure, as reported in some cases (53). In contrast, CD spectra of CHIP28, a water channel found in erythrocytes, solubilized either in octylglucoside micelles or in reconstituted proteoliposomes, were found to be very similar (69). To overcome the dimension problem of micelles with respect to the size of integral membrane proteins, one often synthesizes parts of these proteins that are predicted to be membrane spanning. The synthesis of 28-residue peptides resembling parts of phosphatidylglycerolphosphate synthase was successful in that respect. Their secondary structures in SDS micelles could be well determined by CD studies and confirmed by high-resolution NMR studies (70). Other examples are the synthetic peptides resembling isolated transmembrane helices of BR (71).

2. Membrane-Associated Proteins

When proteins associate with lipid-water interfaces the change in local environment can either destabilize them or introduce structure. This is caused by the difference in helical propensities of amino acids in membrane environments (72) compared with their conformational preferences in water (73). The propensity of proline for β-sheet formation is, however, not affected (74). Both situations, the folding and unfolding of proteins upon interaction with membranes, will be discussed in the next section.

The presence of a preestablished bilayer causes some proteins to adsorb to its interface but others to insert in the membrane. In general, it can be stated that polypeptides that are destabilized on a tertiary and secondary level in aqueous solution exhibit a higher affinity to associate with membranes than the more globular ones do (75). This is best illustrated by apocytochrome c (55), melittin (76), or peptides
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resembling the protein kinase C binding domain of neuromodulin (77) or indolicidin (78). They were all shown to be non-structured in an aqueous environment, but adopted helical (apocytochrome c and melittin) or extended (the protein kinase C binding domain of neuromodulin and indolicidin) structures upon binding to lipid bilayers. However, the binding of more globular proteins to vesicles can also result in an increased helix content, as demonstrated by CD, for example, for glucagon (79) or for rhodanese and its presequence (80).

Numerous studies have been reported on the conformation of water-soluble proteins in the presence of micelles. In some cases detergent concentrations higher than the critical micelle concentration were required to influence the conformational properties of, for example, (apo)cytochrome c (55) or yeast chaperonin protein cpn10 (81). Effects of the acyl chain length of the lipids used on the protein conformation have also been reported, as shown, for example, for myelin basic protein (82). In many cases the effect of the micelle on the structural properties of the associated protein has been shown to be strongly modulated by the nature of the polar lipid headgroups (55,83). The influence of water-micelle interfaces on the structural properties of proteins is best demonstrated in the case of apo- and holocytochrome c, shown in Fig. 14. Whereas in the absence of lipids apocytochrome c is a highly unstructured protein, as indicated by the single negative extreme below 200 nm, the holo protein, which differs only in the presence of a heme group covalently bound to the protein, possesses a CD spectrum having all features of a highly secondary folded protein (Fig. 14). However, after addition of detergent micelles to both proteins an identical CD spectrum is obtained, demonstrating that the water-lipid interface induces a second-

Fig. 14 CD spectra of 0.1 mM apocytochrome c in the absence (a) and presence (b) of dodecylphosphatidylcholine/dodecylphosphatidylglycerol (9:1) micelles and of 0.1 mM cytochrome c in the absence (c) and presence (d) of these micelles in a lipid-to-protein ratio of 120 in a 10 mM phosphate buffer (pH 7.0) (Redrawn from Ref. 60).
ary fold in the apoprotein that is identical to that of the micelle-associated holo protein. From accompanying NMR data it is clear that a common folding intermediate is formed that is a secondary folded but tertiary destabilized protein (60).

B. Proteins at Solid-Air Interfaces

Protein samples can be transferred to a solid carrier in several manners. The most commonly used technique is the Langmuir-Blodgett technique (84). In a Langmuir trough a monolayer is prepared either by self-assembly of proteins at the air-water interface or by protein association with or insertion into a preestablished monomolecular layer of lipids. Subsequent collection of the material on quartz plates is generally achieved by moving the plate through the interface, thereby collecting materials on both sides of the plate. Maneuvering the plate through the monolayer repeatedly can yield a stack of layers on both sides of the plate. The displacement of the barrier of the Langmuir trough allows control of the amount of materials transferred to the plate (85). A more simple and efficient technique is solvent evaporation. The protein solution in the absence or presence of lipids is dried to the air on a quartz plate from an aqueous or organic solvent (86). In the case of pure protein samples, different types and concentrations of alcohol can be added to the solution to control the conformational properties of the adsorbed proteins upon drying of the film (87). In the presence of membranes, spontaneous multilayer arrangements are formed during the evaporation process due to capillary forces that flatten the membranes (86). Dichroism studies using infrared spectroscopy demonstrated that the membrane normal was perpendicular to the plate, yielding a well-oriented lipid-protein system (88). A third approach to transferring proteinaceous materials onto quartz plates is by isopotential spin-dry centrifugation. The solvent is then removed during centrifugation at high gravitational forces (89).

Proteins adsorbed on quartz plates can be placed in a CD spectropolarimeter, provided that the samples are oriented perpendicularly with respect to the incident light beam. Such a setup implies that polypeptides can adopt preferential orientations with respect to the incident light beam, in contrast to randomly dispersed complexes as described earlier. The CD spectrum of a polypeptide in one particular secondary structure type is a superposition of a defined number of (positive and negative) Gaussian absorption bands (90). In helical structures the so-called helix band is also present, which is positive in the long-wavelength region and negative at smaller wavelengths (91). It has to be noted that this latter band has been subject of much controversy (92). The origin of all these bands has been identified [for a review see Woody (93)] and correlated with the direction of absorption in the molecular axis system (56,94–96). Summarizing, two spectra are required to describe the helical contributions of an oriented sample, one corresponding to absorptions of light propagating parallel to the helix axis and the other corresponding to the perpendicular contributions. These two “reference” spectra are depicted in Fig. 15, together with the summation of these two spectra according to \( \theta_{\text{ass}} = (\theta_\parallel + 2\theta_\perp)/3 \), where \( \theta_{\text{ass}} \) represents the ellipticity in an isotropically distributed sample, and \( \theta_\parallel \) and \( \theta_\perp \) represent the parallel and perpendicular contributions, respectively. Figure 15 illustrates that any preferred orientation of helices with respect to the incident light beam does give rise to spectral "distortions." Because all absorptions \( \theta_\parallel, \pi^* \) and \( \pi, \pi^* \) in a \( \beta \)-stranded peptide lie within the same plane parallel to the molecular axis of the \( \beta \)-strand (97,98),
only its CD intensity is affected by a preferred orientation. Analysis of CD spectra of oriented protein systems can be performed in a way similar to that described previously with the CONTIN program but also via nonlinear regression curve-fitting procedures (56), by replacing the reference helix spectrum with the two helical contributions depicted in Fig. 15.

A possible spectral artifact that could interfere with a proper analysis of the protein CD on solid carriers is linear dichroism caused by inhomogeneities of the protein film or the quartz plates used. A general procedure to eliminate this artifact is via rotation of the quartz plate in a plane perpendicular to the incident light beam in cell holders especially designed for this purpose. In such a setup spectra recorded at every 11.25 or 22.5° of rotation are averaged (56,99).

An example of a protein studied at a solid-air interface is the pulmonary surfactant protein (SP-C). It is a small hydrophobic peptide that is palmitoylated at two adjacent cysteine residues. By site-directed mutagenesis these cysteines can be replaced by serines, prohibiting the in vivo acylation. In this way the influence of the acylation on the orientation of the (predominantly helical) peptide at the air-water interface has been studied. A monolayer of this protein was transferred onto quartz plates using the Langmuir-Blodgett technique and studied by CD (100). The palmitoylated SP-C was shown to be oriented preferably parallel to the interface, whereas the nonacylated peptide had a more isotropic orientation. A clear rise of helicity with increasing initial surface pressure of the monolayer was observed, but the preferred orientation was not affected. In another study, monolayers of poly-L-alanine and poly-γ-methyl-L-glutamate spread from different solvents in a
Langmuir trough were transferred to quartz plates and characterized by CD (101). Whereas the first polypeptide was found in each case to be $\alpha$-helical with a strong preferred orientation parallel to the interface, the conformation of the second one could be modulated in either an $\alpha$-helical or a $\beta$-stranded conformation by choice of the (organic) spreading solvent.

Several CD studies have been reported in which the orientation of polypeptides has been determined with respect to oriented membrane systems obtained by evaporation of the aqueous solvent. The most impressive work was presented by Bazzi and Woody (89). They reported on the orientation of the helices of cytochrome $c$ oxidase, which is an integral membrane protein involved in the respiratory chain of mitochondria. An average angle of 39° of the helix axis with respect to the membrane normal was found for this protein. Even more extensive analyses of CD spectra for the preferred orientation of the small peptide alamethicin using oriented membranes have been reported by Vogel (99) and Wu and coworkers (95). Especially in the latter work a comprehensive description of the theory and experimental background of these studies is given. Both studies demonstrated that the orientation of the helix axis of alamethicin is parallel to the membrane normal. The peptide, however, tended to adopt a more perpendicular orientation when the degree of hydration of the membranes was limited. Besides the influence of the degree of hydration, the type of phospholipids differing in net charge or headgroup size was also demonstrated to be of great importance for the orientation of peptides at membrane interfaces (56,102). A method for analysis of oriented CD spectra is described enabling an accurate determination of the average angle that helical structures make with respect to the incident light beam. No knowledge of the actual peptide conformation is required beforehand, and therefore this method is suitable for more general applications to other protein systems (52,102). Using this approach, the domains responsible for the initial insertion of the apocytochrome $c$ into membranes have been identified. The association-insertion behavior of the protein modulated by the composition of the membranes has been described and related to the spontaneous translocation of this protein over the outer mitochondrial membrane. CD on oriented membrane systems has been used more often to determine whether a polypeptide has the expected transmembrane orientation (103) or to study possible conformational changes upon association with monolayers of different composition. For $\beta$-lactoglobulin, for example, it has been shown that it adopts a preferred orientation parallel to the lipid interface (104).

C. Proteins at Solid-Water Interfaces

A few reports can be found in the literature in which CD has been used to establish the structural properties of proteins at solid carrier–water interfaces. Because hydrophobicity of the carrier is of great influence on the protein structure, both hydrophilic and hydrophobic carriers have been used. In the case of small particles stably dispersed in the aqueous phase, the particles are generally nonporous discrete spheres. The particles can be either of silica (hydrophilic) with a diameter ranging from 10 to 20 nm, thereby much smaller than the far-UV CD wavelength (105,106), or of Teflon (hydrophobic). The latter is a copolymer of tetrafluoroethylene and perfluorovinyl ether, has a refractive index close to that of water, and lacks UV-absorbing double bonds (106,107). Both materials are suitable as a sorbent for
using CD to study adsorbed proteins, as they exhibit negligible light absorption and have very low light scattering contributions in far-UV CD. An alternative for these dispersed samples is the use of plane silica quartz plates. They can be made hydrophilic by extensive cleaning in chromic acid and rinsing with distilled water. Alternatively, they can be modified with, for example, C\textsubscript{18} alkyl chains to make them hydrophobic (108). Several quartz plates can be stacked with small spaces in between the plates and incubated with a protein sample in a watertight sample holder. Subsequently, nonadsorbed proteins are removed by replacing the sample buffer by a protein-free buffer (109).

Analysis of CD spectra of adsorbed bovine serum albumin, egg lysozyme, \(\alpha\)-chymotrypsin, and cutinase to hydrophilic silica particles revealed in all cases a loss of secondary structure as compared with the structure of these proteins in aqueous solution (105,106). This is illustrated for cutinase in Fig. 16 by the blue shift of the extreme from 208 to 205 nm and of the zero crossing from 203 to 194 nm upon adsorption to the silica particles. On the other hand, the adsorption of \(\alpha\)-chymotrypsin and subtilisin to hydrophobic particles increased their secondary structure content (106,107). This is illustrated for cutinase by the red shift of the CD zero crossing and 207 extreme in Fig. 16. The specific enzymatic activities of \(\alpha\)-chymotrypsin and cutinase were shown to be correlated with the degree of nativity of the proteins. A similar result was obtained for \(\alpha\)-amylase adsorbed onto silica particles (110). Also, carbonic anhydrase and derived fragments did show a loss of structure upon binding to small silica particles (111). From both far- and near-UV CD data this protein was shown to maintain a secondary fold but to lose its tertiary interactions when adsorbed to this carrier, reflecting a molten globule–like conformation. On the other hand, however, the bee venom peptidolemelittin,

![Graph](image.png)

**Fig. 16** Cutinase in solution (a) and adsorbed onto hydrophobic Teflon particles (b) or onto hydrophilic silica HS-40 particles (c). (Redrawn from Ref. 106.)
known for its ability to adopt under certain conditions amphipatic helical conformations in aqueous solution (112), lost part of its conformation upon adsorption on both the hydrophilic and hydrophobic quartz plates (109). These CD spectra, however, were difficult to analyze because of the spectral distortions due to orientation effects of the adsorbed materials as described earlier.

V. INSTRUMENTAL ASPECTS

The far UV (180–240 nm) is a spectral area in which everything starts to absorb light (also oxygen from air!). In addition, the light sources (usually high-pressure Xe arc lamps) do not have much intensity in this spectral region as compared with the longer wavelengths. One should therefore pay attention to the choice of buffers, salts, protein concentration, and optical path lengths of the cuvettes (1 mm and smaller). Care should also be taken in the preparation of the samples. This is discussed in detail in Refs. 8 and 10. For the near-UV and visible region, normal cuvettes with 1 cm path length are used.

How is circularly polarized light made? Certain electro-optic devices fabricated from, for instance, quartz show changes in birefringence upon experiencing stress caused by a voltage between two opposing faces of a block. Such a device is called a photoelastic modulator. The effect of a so-called quarter-wave plate causing birefringence is clarified in Fig. 17. Such an optical device is made from a crystal with the direction of the optic

![Diagram](a)

**Fig. 17** Generation of circularly polarized light from plane-polarized light by a quarter-wave plate (a). The generation of left and right circularly polarized light by a photoelastic modulator (b).
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axis as indicated. Plane-polarized light is represented in the figure by two plane-polarized waves perpendicular to each other. The component that has the electrical field component parallel to the optic axis propagates faster through the crystal than the perpendicular component does. The latter is retarded because it senses a higher refractive index. The outgoing beam consists of two perpendicular waves that are 90° out of phase (the thickness of the crystal is chosen such that one wave is one-quarter wavelength ahead of the other wave), which is, bearing Fig. 2d in mind, equivalent to circularly polarized light of a given handedness. Upon applying stress to such electro-optic material, the optic axis changes by 90° (called birefringence). In this way the other circularly polarized light wave is created. By modulating the voltage (which induces fluctuating birefringence) with a fixed frequency (for instance 20 kHz), alternately left and right circularly polarized light is created (Fig. 17). Figure 18 shows a simplified block diagram of a single-beam CD spectrometer.

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**Fig. 18**  (a) Block diagram of a CD spectrometer. (b) Illustration of the tiny dichroism effect to be measured. $A_L$ and $A_R$ are the absorbances for left and right circularly polarized light. The factor $10^{-4}$ illustrates that the signal related to the differential absorbance is $10^{-4}$ times smaller than the normal absorbance signal.
The extinction differences (Δε) observed in a CD spectrometer are extremely small (Fig. 18). They are measurable only using phase-sensitive detection techniques. The signal coming from the detector has two components. One component is a DC signal (V_Dc), which is related to the overall light throughput. The other AC component (V_AC) contains the differential absorption information ΔA (ΔA = Δε/(C.d)), with C the concentration in mol dm⁻³ and d the optical path length in cm:

$$\Delta A = k \frac{V_{AC}}{V_{DC}}$$

(15)

in which k is an instrumental constant. The synchronous detection of the signal relative to the modulation frequency of the photoelastic modulator determines V_AC. Both V_AC and V_DC are amplified (or attenuated) by an identical factor using a servo system in such a way that V_DC remains constant. The circular dichroism effect expressed in ΔA is

$$\Delta A = k' V_{AC}$$

(16)

with k' a simple calibration factor that can be set by the electronics.

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