ENZYME LIGAND COMPLEXES: SPECTROSCOPIC STUDIES

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INTRODUCTION

The various forms of visible and ultraviolet (UV) spectroscopy (including difference spectral measurements, circular dichroism, optical rotatory dispersion, and fluorescence spectroscopy) have become very useful tools for examining protein-ligand interactions. These interactions include the binding of substrates and inhibitors to enzymes and the association of drugs with receptor proteins and other serum and tissue proteins. In favorable circumstances the spectroscopic methods may be used to directly determine the binding constants of the protein-ligand complexes. In addition, these techniques may give insight into the factors which influence the binding. For example, changes in the UV or visible spectrum of a ligand upon binding to a protein may indicate the polarity of the binding site or illustrate changes in the degree of ionization of the ligand upon binding. In addition, specific environmental changes of amino acid residues may be detected by changes in the protein absorption spectrum. Circular dichroism and optical rotatory dispersion often yield information about the types of amino acids involved in the binding process, and these techniques have also been employed to examine the probable conformation of the ligand when bound to the surface of the protein. Fluorescence spectroscopy is a very sensitive tool. Fluorescence measurements have been used to obtain binding constants, to demonstrate structural changes upon ligand binding, and to estimate binding distances between two fluorescent chromophores on a protein.

In this review several numerical methods of determining equilibrium constants of protein-ligand complexes from spectral data will be discussed, and the advantages and pitfalls of some of the various methods will be examined. The various types of spectroscopy will be briefly described, and several examples from the literature utilizing each method will be discussed. Unfortunately, the literature on spectroscopic methods spans several decades and, thus, a complete review of the literature is beyond the scope of this review. As usual, in a review of this nature, the references cited will be prejudiced by research interests of the reviewer, and the author apologizes to the researchers whose works are not mentioned.

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DETERMINATION OF EQUILIBRIUM CONSTANTS

Spectroscopic methods may be used to determine binding constants of ligands to proteins when the binding of the ligand is accompanied by a change in one of the physical properties of the protein which is proportional to the amount of bound ligand. This change may be in the UV spectrum of the ligand and protein compared to the unmixed components, in optical rotation or circular dichroism associated with complex formation, or in the fluorescence intensity (either enhancement or quenching) upon mixing.[1] The theory of the measurement of molecular complexes has been discussed in detail by several authors.[2-5]

One Binding Site

When an enzyme or other protein has only one binding site for any particular ligand, spectral measurements may be easily used to determine binding constants. Using the notation of Deranleau,[4] the binding constant, k, of a molecular complex is commonly determined from measurement in which a dilute component, P (e.g., protein), with a total concentration, P_0, is titrated by the addition of a second component, X (e.g., substrate, inhibitor, metal ion), with total concentration, X_0. When a 1:1 complex, PX, is formed to the exclusion of all other complexes, the formation constant is defined by:

$$[PX] = k[P][X] = k([P_0]-[PX])/([X_0]-[PX]) \quad (1)$$

$$\text{or } k = \frac{[PX]}{[P][X]}$$

The saturation fraction, s, is defined as

$$[PX]/[P_0] \text{ or } s = \frac{k[X]}{(1+[X])} \quad (2)$$

This saturation fraction is the probability of binding. If A is the difference in absorption (or fluorescence or circular dichroism, etc.) of a given mixture of protein and ligand minus the absorption of the unmixed components and ϵ is the proportionality constant, $A = \epsilon[PX]$. When all of X is complexed the absorbance due to the complex will have a maximum value $A_{max} = \epsilon[P_0]$. The saturation fraction is, therefore,

$$s = A/A_{max} = A/\epsilon[P_0] = [PX]/[P_0] \quad (3)$$

Weak Molecular Complexes

The determination of the binding constant of a weak molecular complex has been reviewed in detail by Deranleau.[4] In this case one can choose conditions where the concentration of free (unbound) ligand X is large compared to the concentration of the complex. One can thus make the substituted $[X] = [X_0]$ within a specified error. This error is $(k' - k)/k = -s[P_0][X_0]$ where k' is the value calculated using the approximation $X = X_0$ and k is the value calculated from the exact equation where $[X] = [X_0] - [PX]$.

There are several graphical techniques to plot spectroscopic data to yield binding constants. Deranleau[4] points out that in order to fit any data to a model to obtain constants with physical significance it is necessary to be able to plot data which cover at least 75% of the saturation curve. Deranleau suggests that the best ways to represent binding data for weak complexes are: (1) the direct plot of $A/[P_0]$ vs. $[X]$; (2) the plot of $A/[P_0]$ vs. $\log[X]$ (i.e., the titration curve);[6] (3) the plot of $A/[P_0][X]$ vs. $A/[P_0]$ (i.e., the Scatchard plot).[7] This third method plots data according to the equation

$$\frac{A}{[P_0][X]} = k\left(\epsilon - \frac{A}{[P_0]}\right) \quad (4)$$

The slope of the line is thus $-k$ and the y intercept is $k\epsilon$. These methods represent the whole titration curve, identifying what portion of the saturation curve one has covered. The three methods of portraying data are shown in Figure 1.

One may also determine the binding constant by plotting the data in reciprocal plots such as $1/A$ vs. $1/[X_0]$.[8] In this case the y intercept yields $1/A_{max}$ and the x intercept $-k$; however, Deranleau[4] cautions against these plots because only a small portion of the binding curve need be represented, and the constants one obtains may, therefore, be very misleading.

Strong Molecular Complexes

When the concentration of the free component, $[X]$, is of the same order of magnitude as the concentration of the complex, $[PX]$, one may no longer estimate the concentration of $[X]$ by $[X_0]$, but must make the substitution $[X_0] - [PX] = [X]$ or estimate the concentration of $[X]$ by

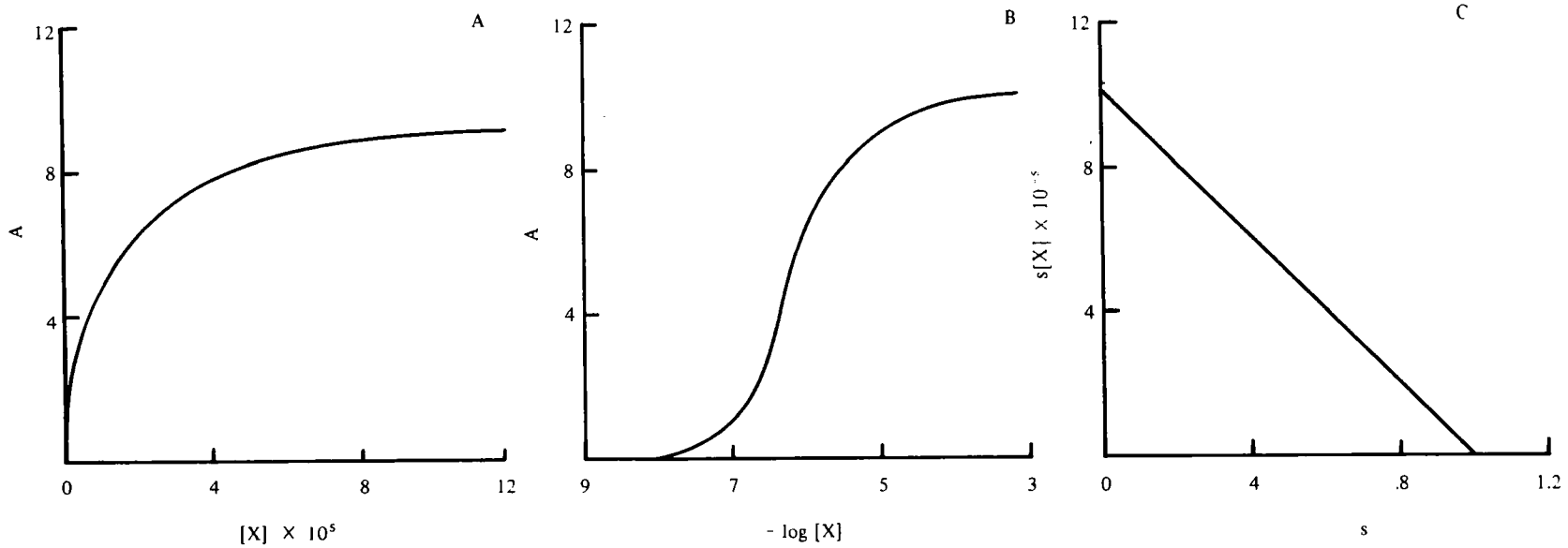


FIGURE 1. Methods of portraying spectroscopic enzyme-ligand binding data. (A) The direct plot; (B) The titration curve; (C) The Scatchard plot. Theoretical curves assume a binding constant of 10^6 *M*, an enzyme concentration (c) of 10^{-5} *M*, and an extinction coefficient (ϵ) for the change due to complex formation of 10^6. The absorbance of the enzyme-ligand complex is A. $s = A/(c \times \epsilon)$.

nonspectroscopic means such as equilibrium dialysis or potentiometry.

The concentration of the complex [PX] may be easily determined by rearranging Equation 3 if one knows the concentration of protein $[P_o]$ precisely and can easily measure A_{max}. These values, however, are often difficult to obtain. Recently several methods have been described to obtain estimates of these values from purely spectroscopic methods.

Kurganov et al.[9,10] have described a "difference" method for the estimation of binding constants of enzyme-ligand complexes from fluorometric data. The method can be applied, however, to any type of spectroscopic method. From Equation 3 when [PX] is equal to 0, A is 0, and when [PX] equals P_o, $A = A_{max}$. At the ith total ligand concentration X_{oi} with total protein concentration P_o,

$$PX_i = P_o\ (A_i/A_{max}) \tag{5}$$

Putting Equation 5 into the definition for k, along with the conservation of mass equation, gives

$$k = \frac{(A_i/A_{max})}{(1-A_i/A_{max})\ ([X_{oi}]-(A_i/A_{max}))} \tag{6}$$

For two different total ligand concentrations, X_1 and X_2, at the same total enzyme concentration the right-hand side of Equation 2 is equal for $X_{oi} = X_1$ and $X_{oi} = X_2$; therefore,

$$\frac{(A_1/A_{max})}{(1-(A_1/A_{max}))\ (X_1-P_o\left(\frac{A_1}{A_{max}}\right))} = \frac{(A_2/A_{max})}{(1-\left(\frac{A_2}{A_{max}}\right))\ (X_2-P_o\left(\frac{A_2}{A_{max}}\right))} \tag{7}$$

Rearranging terms one obtains

$$\frac{\left(\frac{[X_1]}{A_1}-\frac{[X_2]}{A_2}\right)}{([X_1]-[X_2])} = \frac{1}{A_{max}} + \left(\frac{P_o}{(A_{max})^2}\right)\ \left(\frac{A_1-A_2}{[X_1]-[X_2]}\right) \tag{8}$$

By setting

$$x = \left(\frac{A_1-A_2}{[X_1]-[X_2]}\right) \quad \text{and} \quad y = \frac{\frac{[X_1]}{A_1}-\frac{[X_2]}{A_2}}{([X_1]-[X_2])} \tag{9}$$

for a series of pairs of X_{oi} and X_{oi+n}, these equations define a straight line where the intercept on the y axis = $1/A_{max}$ and the slope is equal to the protein concentration, P_o, divided by the square of A_{max}. Once these parameters are obtained k can easily be estimated at any point from Equation 6.

The method of Kurganov et al.[9,10] is very useful when the concentrations of P_o and A_{max} are unknown. The data must be relatively precise, however, as the constants are obtained from differences between paired points, and these differences may be very small. Their method of portraying data is shown in Figure 2.

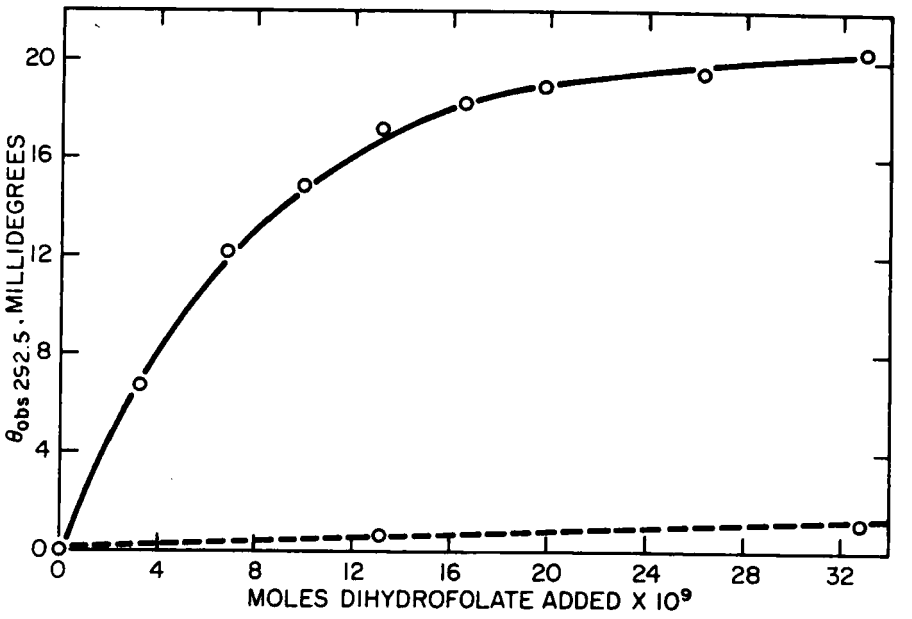
Another differential type of measurement to obtain the concentration of X when the approximation $[X_o] = [X]$ is invalid has recently been described by Halfman and Nishida.[11] To use this method one must know the protein concentration, but A_{max} need not be evaluated. The number of equivalents of bound ligand and the concentration of free ligand are computed from data obtained at a minimum of two protein concentrations. In the method of Halfman and Nishida[11] data are plotted as A vs. $[X_o]/[P_o]$. As shown above,

$$[X] = ([X_o]-[PX]) \text{ or}$$
$$[X] = [P_o](([X_o]/[P_o])-s) \tag{10}$$

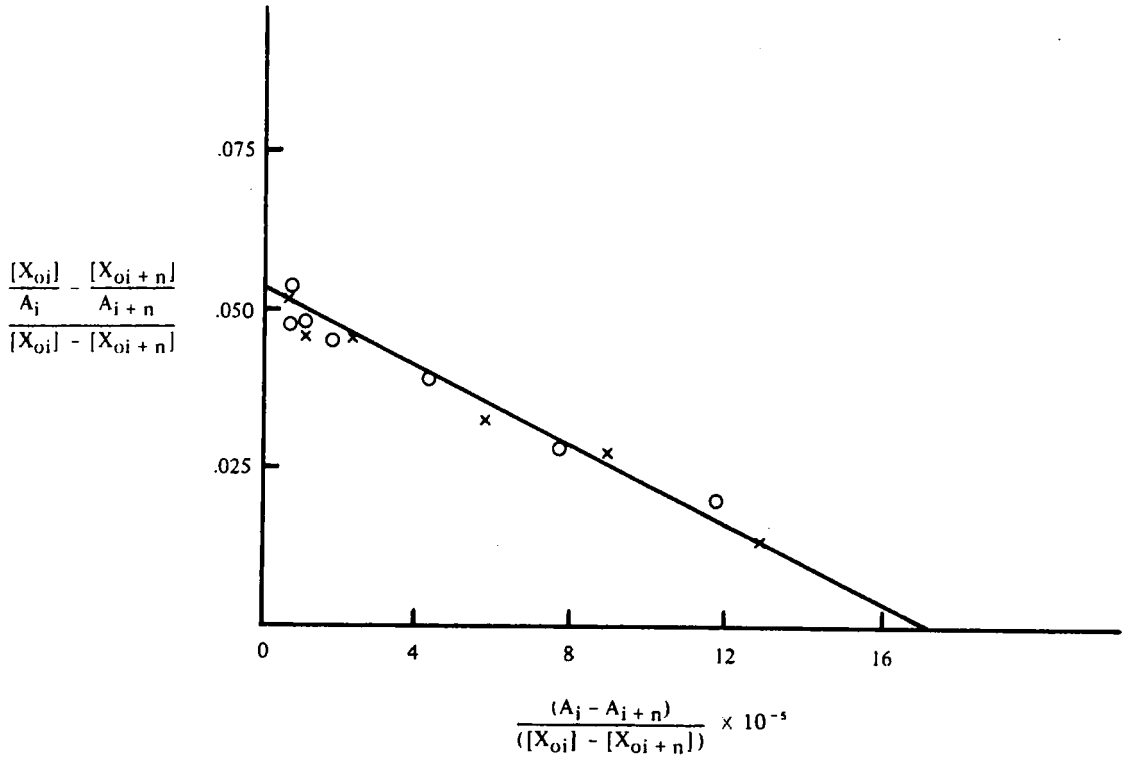
When data are collected and plotted at two different protein concentrations, $[P_1]$ and $[P_2]$, it follows that

$$s = \frac{\left(\frac{X_o}{P_1}\right) - \frac{[P_2]}{[P_1]}\left(\frac{X_o}{P}\right)_2}{\left(1-\frac{[P_2]}{[P_1]}\right)} \tag{11}$$

and



A



B

FIGURE 2. The determination of the binding constant of the complex of dihydrofolate reductase with dihydrofolate using the "difference" method of Kurganov et al.[9] from circular dichroism data of Greenfield et al.[28] (A) CD titration – solid line = enzyme + dihydrofolate; dashed line = dihydrofolate alone. Titration of 0.9 ml of enzyme with dihydrofolate; (B) Difference plot – (o-o) n = 1, (x-x) n = 2. From the difference plot, the enzyme concentration is 1.07×10^{-5} *M*, A_{max} = 19.9 millidegrees, $K_d = 1.2 \pm 0.1 \times 10^{-6}$ *M*. (From Greenfield, N. J., Williams, M. N., Poe, M., and Hoogsteen, K., *Biochemistry*, 11, 4706, 1972. With permission.)

$$[X] = \frac{[P_1]\ [P_2]}{[P_1]-[P_2]}\left[\left(\frac{X_o}{P}\right)_2 - \left(\frac{X_o}{P}\right)_1\right] \qquad (12)$$

where

$(X_o/P)_1$ = total equivalent of ligand added at protein concentration $[P_1]$;
$(X_o/P)_2$ = total equivalent of ligand added at protein concentration $[P_2]$.

The equations are obeyed when the addition of $(X_o/P)_1$ and $(X_o/P)_2$ give the same value of A corresponding to a particular value of s. From these values one can construct the Scatchard plot of s/[X] vs. s where the y intercept will directly give the binding constant, k. Again, as the method depends on taking the difference of several pairs of data, it is necessary that the raw spectral data be highly accurate and that the binding constant be independent of protein concentration. Various graphical methods of obtaining binding constants are shown in Table 1.

The Scatchard plot,[7] the difference method of Kurganov et al.,[9,10] and the method of Halfman and Nishida,[11] when applied to one binding site, are means of transforming data so that a straight line plot can be used to estimate the binding constant. In the three treatments a "linear regression analysis" (i.e., the method of least squares) can be used to find the best fit to the data. With the availability of computers increasing, however, there are advantages in fitting the binding curve directly. Such fitting of the data eliminates some of the errors introduced by pairing found in the methods of Kurganov et al.[9,10] and Halfman and Nishida,[11] and also allows the direct determination of the A_{max} which gives the best fit to the data without extrapolations. Direct fitting also does not weigh the data unevenly.

In the special case of one binding site there are three parameters which one might wish to determine from the binding data. These are A_{max}, P_o, and k. Several methods of determining these values have recently been published; these may be of interest to the enzymologists and protein chemists who are not expert in the use of computers.

The first method, described by Hoare,[12] is a general means of utilizing a small computer for solving equations containing three or four unknown constants. In Hoare's approach one must express the binding curve as a function of an independent variable (in this case X_{oi}, the total ligand concentration at any point), a dependent variable (in this case A_i, the measured physical change at that point), and constants which relate A_i to X_{oi} (e.g., A_{max} P_o, and k). This relationship is established by solving Equation 6 for A_i. One then estimates the values of the constants, calculates the A_i that would be obtained using these constants for any X_{oi}, and then calculates the error between the calculated A_i's and the observed A_i's. The error, S, is shown below:

$$S = \sum_{i=1}^{n} \frac{(A_{oi} - F(k, P_o, A_{max}, E_{oi}))^2}{n} \qquad (13)$$

or the weighted error

$$S = \sum_{i=1}^{n} \frac{(1 - (F(k, P_o, A_{max}, E_{oi})/A_{oi}))^2}{n} \qquad (14)$$

The most probable values of k, P_o, and A_{max} are the values which minimize S. Hoare[12] gives methods of finding the minimum S value when up to four constants are unknown. Using the method of Hoare, if the binding curve contains only one unknown (e.g., k), but A_{max} and $[P_o]$ are known, one would list a range of values of k and solve Equation 6 for A_i at every point. One would find the error between the calculated A_i's and the experimental A_i's and plot the sum of squares of the calculated and experimental A_i's against the assumed k. This plot is called a sum of squares curve or S curve. The most probable value of k would be found from the minimum in the S curve.

When there are two unknowns (e.g., k nd P_o) the computer can be used to calculate the values of S corresponding to pairs of P_o and k and print out these values in a matrix relating S to P_o and k. Contours joining equal values of S can be drawn onto this matrix to indicate the shape of a surface whose height at any point of P_o and k gives the value of S corresponding to these values of P_o and k. This surface is known as a sum of squares surface or S-surface. The values of P_o and k which give the minimum S are the most probable values. Hoare[12] also gives a method of estimating the confidence in the values of the determined constants using Equation 15,

$$S(x\%) = S(\text{minimum})\left(1+\frac{p}{n-p} \cdot f(p, (n-p), x\%)\right) \qquad (15)$$

where

TABLE 1

Graphical Methods of Obtaining Binding Constants: One Binding Site

Name	Known	Unknown	Procedure
Scatchard plot	$[P_o]$, $[X_{oi}]$ A_i, A_{max}	k	Plot $A_i/([P_o]([X_{oi}] - A_i/A_{max}))$ vs $A_i/[P_o]$ slope = $-k$, y intercept = $k\epsilon$
Difference method I	$[X_{oi}]$, A_i	$[P_o]$, A_{max}, k	Plot $(([X_{oi}]/A_i) - ([X_{oi+n}]/A_{i+n}))/([X_{oi}] - [X_{oi+n}])$ vs $(A_i - A_{i+n})/([X_{oi}] - [X_{oi+n}])$ slope = $P_o/(A_{max})^2$, y intercept = $1/A_{max}$ $k = [\sum_{i=1}^{N}[(A_i/A_{max})/((1 - A_i/A_{max})([X_{oi}] - A_i/A_{max}))]/N$
Difference method II	$[P_o]$, A_i, $[X_{oi}]$	k, A_{max}	Plot A_i vs $[X_{oi}]/P_o$ for at least two protein concentrations $[P_1]$ and $[P_2]$ Let $s = ((X_o/P)_1 - ([P_2]/[P_1])(X_o/P_2))/(1 - [P_2]/[P])$ Let $[X_i] = ([P_1][P_2]/([P_1] - [P_2]))((X_o/P_2) - (X_o/P_1))(X_o/P)_1))$ where $(X_o/P)_1$ and $(X_o/P)_2$ are the total equivalents of ligand added at total protein concentrations $[P_1]$ and $[P_2]$ which give the same value of A_i; plot $s/[X_i]$ vs s, k = y intercept
Benesi-Hildebrand plot	$[X_i]$, A_i	k, A_{max}	Plot $1/A_i$ vs $1/[X_i]$ y intercept = $1/A_{max}$, x intercept = $-k$

Symbols:

$[P_o]$ = total protein concentration;
$[X_{oi}]$ = total ligand concentration at any point, i;
$[X_i]$ = free ligand concentration at any point, i;
A_i = spectral change at any point, i;
A_{max} = spectral change when total protein is complexed;
k = association constant;
n = distance between different points on curve of A_i vs $[X_{oi}]$;
N = number of points;
ϵ = extinction coefficient of spectral change associated with complexation.

x% = confidence level;
p = number of constants to be evaluated;
r = number of data points;
f( ) = value from tables for the F-test for equality of variances.

The degree of confidence is only approximate, as the equation of binding is nonlinear.

Hoare[12] gives methods of applying S-surfaces when there are up to four unknowns. In the procedure the S-curves and S-surfaces are constructed so that at every point the value of S is minimized with respect to the constants that do not appear in the printout. For example, if k, A_{max}, and P_o are all unknown, one may assume values of A_{max} and P_o and calculate k from Equation 6; then one may use the calculated best k which fits the data given A_{max} and P_o and then proceed as above.

A method similar to that described by Hoare was used by Gammon et al.[13] to find the binding constant of *N*-trifluoroacetyl-D (and -L) *p*-fluorophenylalanine to chymotrypsin. Their method was developed for nuclear magnetic resonance data, but can easily be applied to optical spectroscopy. In this method one must know $[P_o]$ independently. To use their method: (1) assume a value of k; (2) calculate [PX] at every point using the equation $[PX] = ([P_o] + [X_o] + 1/k - \sqrt{([P_o] + [X_o] + (1/k))^2 - 4[P_o][X_o]})/2$; (3) obtain the best linear least squares fit between the computed values of [PX] and the experimental values of A from Equation 5, (i.e., plot A vs [PX]); (4) sum the error between the straight line determined by the least squares fit of Step 3 and the actual points. Repeat Steps 1 to 4 assuming different values of k. The final value of k is that which gives the smallest error in Step 4, and the slope of this line is taken as P_o/A_{max}. (In practice one plots k_i against the error for a wide range of k_i's and then takes smaller intervals between the k_i's giving the lowest minimum errors.) The method is illustrated in Figure 3.

The methods of Hoare[12] and Gammon et al.[13] are basically search procedures where one examines a range of values of unknown constants to arrive at the values which give the best fit to the data. Recently Engel[14] has described an iterative procedure for finding the association constant (k), the number of binding sites (n), and the theoretical titration endpoint (A_{max}) of an enzyme-ligand interaction monitored by fluorescence quenching. Once again, his method is applicable to any spectroscopic procedure.

The calculation of the theoretical fluorescence quenching curve is based on Scatchard's equation:

$$s = \frac{n k [X]}{1 + [X]} \tag{16}$$

As shown above $s = A/A_{max}$, and by substitution one obtains

$$A = A_{max}\left[\frac{1+nk[X_o]+k[P_o]}{2k[P_o]} - \left(\left\{\frac{1+nk[X_o]+kP_o}{2kP_o}\right\}^2 - \frac{nX_o}{P_o}\right)^{\frac{1}{2}}\right] \tag{17}$$

Engel[14] makes initial estimates of n, k, and A_{max} and uses a linear Taylor series approximation to improve the estimates. Engel has stated that his computer program is available upon request. The mathematical analysis is too lengthy to restate here, but is written up clearly and simply in Engel's manuscript. The method has the advantages of not weighing the data unevenly, not requiring the prior knowledge of the endpoints, and not relying on taking differences which may introduce errors; however, the initial estimates of k, n, and A_{max} must be good if the series is to converge.

Multiple Binding Sites

When a protein has only one binding site for a ligand and the binding of the ligand is accompanied by a physical change, there are several straightforward methods for determining the binding constant as shown above. When a protein has multiple binding sites, however, the situation becomes much more complicated, and often spectral data alone will not provide enough information to determine binding constants uniquely.[5,15-17]

The theory of multiple equilibrium has been treated by several authors.[1-3,18] If a molecule contains n reactive sites where a ligand, X, can bind to a protein, P, the binding can be characterized by n association constants where:

$$k_1 = \frac{[PX]}{[P][X]} \qquad k_2 = \frac{[PX_2]}{[PX][X]} \qquad k_i = \frac{[PX_i]}{[PX_{i-1}][X]} \qquad k_n = \frac{[PX_n]}{[PX_{n-1}][X]} \tag{18}$$

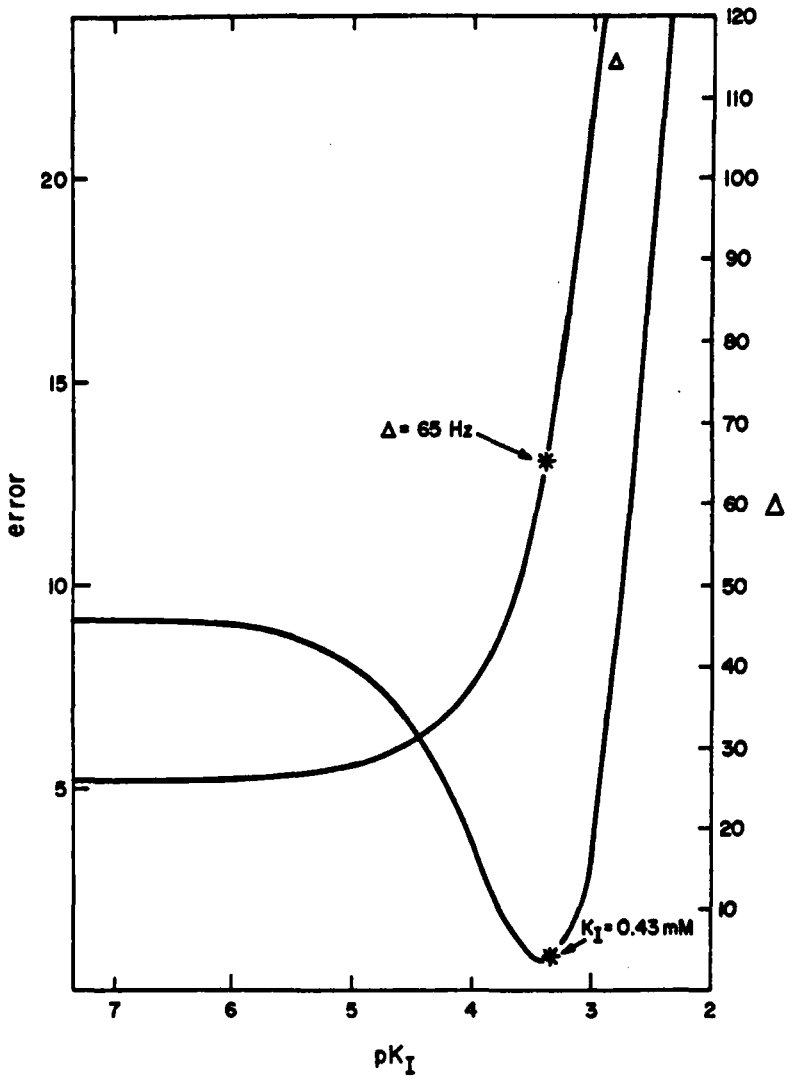


FIGURE 3. The determination of the dissociation constant of an enzyme-inhibitor complex (K_I) and spectral extinction coefficient ($A_{max} = \Delta$) by finding the parameters which give the minimal error between the calculated and observed data. (From Gammon, K. L., Smallcombe, S. H., and Richards, J. H., *J. Am. Chem. Soc.*, 94, 4573, 1972. With permission.)

PX indicates the sum of the concentrations of all the n microscopically different species in which one X molecule is bound to any P, and PX_i denotes the sum for all the species in which i of the n sites are occupied by X.

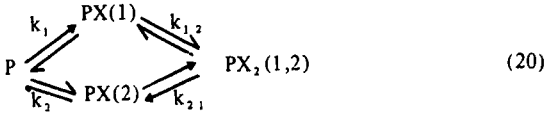
Spectral measurements can become quite complicated as the binding of X to the protein at site 1 may induce quite a different effect than when X binds to the protein at site 2 (or 3, etc.). Moreover, if site 1 is occupied, the binding of X at site 2 may induce a different effect than if site 1 is empty.

If all the sites are equivalent and independent so that the reaction at each site may be characterized by a single intrinsic microscopic constant which is the same for all the sites, then

$$K_1 = nk \qquad K_2 = (n-1)k/2 \qquad K_3 = (n-2)k/3 \qquad \cdots \qquad K_n = k/n \tag{19}$$

In this special case Equation 16 holds, where n is the number of binding sites and k is the intrinsic binding constant.

Deranleau[5] has discussed the situation when there are only two binding sites in great detail. There are two possible pathways by which the enzyme may bind a ligand, as shown below.



(20)

If $k_1 = k_2 = k_{12} = k_{21}$, then the sites are equivalent and independent. If $k_{12} = k_2 < k_1 = k_{21}$, this is normal noncooperative binding. If all the equilibrium constants are distinct, there may be competitive or cooperative binding and $k_1 \neq k_2 \neq k_{12} \neq k_{21}$. The absorbance of a solution of protein and ligand is

$$A = \epsilon(1)\,[PX(1)] + \epsilon(2)\,[PX(2)] + \epsilon(1,2)\,[PX_2(1,2)] \quad (21)$$

assuming that Beer's law hold for each species. The total concentration of protein is

$$[P_O] = [P] + [PX(1)] + [PX(2)] + [PX_2(1,2)] \quad (22)$$

and the association constants are

$$PX(1) = k_1[P]\,[X] \qquad PX(2) = k_2[P]\,[X]$$

$$PX_2(1,2) = k_{12}\,[PX(1)]\,[X] = k_1k_{12}[P]\,[X]^2 = k_{21}[PX(2)][X] = k_2k_{21}[P]\,[X]^2 \quad (23)$$

Eliminating P from the equations gives

$$\frac{A}{P_O} = \frac{(k_1\epsilon(1) + k_2\epsilon(2))\,[X] + k_1k_{12}\epsilon(1,2)\,[X]^2}{1 + (k_1 + k_2)\,[X] + k_1k_{12}\,[X]^2} \quad (24)$$

The above expression is for microscopic extinction coefficients; that is, each site has its own extinction coefficient. In practice these coefficients may be impossible to determine. The macroscopic constants are $K_1 = k_1 + k_2$ and $K_1K_2 = k_2k_{21}$, and the weighted average macroscopic extinction coefficients are

$$\epsilon_1 = [k_1\epsilon(1) + k_2\epsilon(2)]/(k_1 + k_2)\quad \epsilon_2 = \epsilon(1,2) \quad (25)$$

Substituting one gets

$$\frac{A}{[P_O]} = \frac{K_1\epsilon_1[X] + K_1K_2\epsilon_2[X]^2}{1 + K_1[X] + K_1K_2[X]^2} \quad (26)$$

The problem is to determine the equilibrium constants from the spectroscopic data. As one can see, the expression is quite complicated even when there are only two binding sites.

When both binding sites have the same binding constant and the same extinction coefficient, as in the case $K_1 = 4K_2 = 2k$ and $\epsilon_2 = 2\epsilon_1$, the expression simplified to

$$\frac{A}{2\epsilon[P_O]} = \frac{k[X]}{1 + k[X]} = s \quad (27)$$

Except for the factor of 2 in the denominator, the expression is the same as the binding equation for one binding site and, indeed, all the methods described for one binding site can be used to determine the binding constants for multiple binding sites under these conditions. In the case of independent equivalent sites the methods of Kurganov et al.,[9,10] Halfman and Nishida,[11] and Engel,[12] will yield the number of binding sites as well as the microscopic binding constant, provided that the enzyme (or protein) concentration is known.

If the binding sites of ligands are not independent and equivalent, the determination of equilibrium constants from spectroscopic data may be quite difficult. Indeed, in some cases of multiple equilibria, it is impossible to determine equilibrium constants from the data because there will be many good fits to the data. This problem of redundancy has been discussed in detail by Reich and co-workers.[15-17]

In the special case where there are only two binding sites or two classes of binding sites, several treatments of spectroscopic data may be of use.

Weak Binding (Two Binding Sites)

The special case of weak binding of ligands to two binding sites has been discussed by Deranleau.[5] Then binding data is plotted in a Scatchard Plot[7] as $A/[P_O][X]$ vs $A/[P_O]$. The slope at the low saturation limit as $X_o \rightarrow 0$ is $-(K_1 - K_2\epsilon_2/\epsilon_1)$ and the intercept is $K_1\epsilon_1$. The slope at

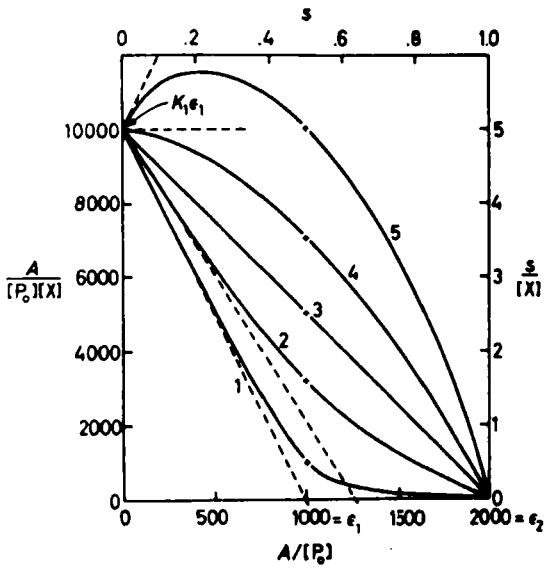


FIGURE 4. Scatchard plots of various types of multiple equilibria. Curves 1 and 2 are ordinary noncooperative equilibria. Curve 3 is the straight line obtained where all the constants are identical. Curves 4 and 5 show cooperative effects. (From Deranleau, D. A., *J. Am. Chem. Soc.*, 91, 4050, 1969. With permission.)

the high saturation limit as $s \to 1$ and $[X_0] \to \infty$ is $-K_2(1 - \epsilon_1/\epsilon_2)^{-1}$ and the intercept is ϵ_2. Deranleau[5] points out that unless $K_1\epsilon_1 >> K_2\epsilon_2$, unique values of both constants will be almost impossible to obtain. The Scatchard plot for two variables is shown in Figure 4.

Relatively Strong Binding (Two Classes of Binding Sites)

The method of Halfman and Nishida[11] described for one binding site may also be used to determine a Scatchard plot from spectral data in the case of multiple binding sites, provided that the ligand binding is independent of protein concentration. Halfman and Nishida also give methods for determining n_1, n_2, K_1, and K_2 if the plot can be described by two classes of sites.

The method described by Hoare[12] may also be applied to cases where there are two binding sites in some special cases. For example, if the binding is relatively tight and there are two binding sites, the macroscopic extinction coefficient for the first site, ϵ_1, may be determined by titrating samples of ligand with protein at several ligand concentrations. The limiting change in absorbance (or fluorescence, etc.) at high protein concentration corrected for the absorbance of total ligand and protein if they were unbound, divided by the concentration of ligand is ϵ_1. The macroscopic extinction coefficient of protein species with both sites filled, ϵ_2, may be determined by titrating samples of protein with ligand at several protein concentrations. The limiting change in absorbance at saturation (once again corrected for the absorbance of the ligand and protein as if they were unbound) divided by the total protein concentration is ϵ_2. If there is only one binding site for a ligand upon a protein, $\epsilon_1 = \epsilon_2$. If there are multiple nonequivalent binding sites, then $\epsilon_1 \neq \epsilon_2$. If there are no equivalent binding sites for the ligand, $\epsilon_2 = n\epsilon_1$. (This treatment has been adopted from Williams et al.)[19]

Once the extinction coefficients are determined, the binding equations may be rewritten in their macroscopic form.

$$K_1 = \frac{[PX]}{[P][X]} \tag{28}$$

$$K_2 = \frac{[PX_2]}{[PX][X]} \tag{29}$$

The equations for the equality of mass are

$$[P_0] = [P] + [PX] + [PX_2] \tag{30}$$

$$[X_0] = [X] + [PX] + 2[PX_2] \tag{31}$$

and the equation for the change in absorbance (etc.) is

$$A = \epsilon_1 PX + \epsilon_2 PX_2 \tag{32}$$

To find the best fit to the binding data it is necessary to find K_1 and K_2, which give the theoretical spectral data closest to the observed spectral data at every point for a series of A_i as a function of X_{0i}. One method of doing this would be to assume K_i and calculate [PX] at every point using Equations 31, 32, and 28. This is a quadratic equation. Once [PX] is determined, $[PX_2]$ can be determined from Equation 32 and [X] can be found from Equation 31; then K_2 may be determined from Equation 29, and K_2 averaged for all the points. Once K_1 and K_2 are determined, it is possible to solve for [X], given $[X_0]$, using Equations 28 through 31 (it is a cubic equation), and thus solve for A, the absorbance at any given X_0, using Equation 26. One can then calculate the deviation between the calculated absorbance

change and the observed absorbance change at any point. If one repeats this process for a series of K_1's, one can construct the S-curve as described by Hoare.[12] The best fit of the data will be the K_1 and K_2 which give the minimum error.

In theory it should be possible to determine binding constants even when there are more than two sets of binding sites. In practice solutions may become quite complicated and there may be several suitable fits to the data, making the determination of true binding constants difficult, if not impossible. There are several treatments of the problem of multiple binding sites which may be of interest to those trying to fit spectral data to theoretical binding curves. These include Reich and co-workers,[15-17] Thompson and Klotz,[20] Klotz,[21] and Fletcher and co-workers.[22-24] Several treatments of finding solutions to non-linear equations may also be of use,[25,26] but they are beyond the scope of this paper.

In all spectral measurements there is one caveat. It is impossible to prove the number of binding sites from spectral data alone. While it is obvious that in the case of weak binding there may be several fits to the data, as discussed by Deranleau,[4,5] this is not as obvious in the case of strong binding. However, if the binding constants are well separated and the extinction coefficients are also different, the spectral data may be quite misleading. For example, fluorescence measurements show two binding sites for 4-butyl(*p*-nitrophenyl)-2-phenyl-3,5-pyrazolidinedione to human plasma albumin when the data are fitted by a Scatchard plot and the data fit a straight line; however, equilibrium dialysis measurements show additional binding sites.[27] Similarly, the circular dichroism titration of dihydrofolate reductase from *E. coli B* (MB 1428) in the presence of methotrexate shows one stoichiometric binding site[28] for TPNH while equilibrium ultrafiltration and Sephadex filtration binding experiments show two sites.[29] The binding at the second site is moderately tight, but the binding is weaker than at the first; thus, the binding of the second mole has no effect on the circular dichroism of the complex. Supplementary data (in the form of some sort of direct measurement, such as equilibrium dialysis) are always necessary for the interpretation of spectral data.

DIFFERENCE SPECTROSCOPY

The binding of a substrate or an inhibitor to a protein often causes changes in the UV spectrum of the complex compared to the sum of the spectra of the unmixed components. These changes may be due to perturbation of the chromophores of the proteins or shifts in the spectrum of the ligand due to changes in its environment or state of ionization. In addition, new bands may appear in the spectrum of the enzyme-ligand complex due to charge transfer. The spectral perturbations caused by enzyme-ligand binding have several uses. First, as shown above, the spectral changes can be used to measure enzyme-ligand interaction. Second, the changes can sometimes be interpreted to yield useful information about the nature of the protein-ligand interaction. The use of UV spectroscopy in the study of protein chemistry has been reviewed by several authors.[29-38]

Often, the spectral perturbations caused by enzyme-substrate interactions are very small compared to the spectrum. In order to visualize the changes easily, it is useful to examine the spectrum of the mixed components compared to that of the unmixed components. This technique is called difference spectroscopy and was popularized by Bastian,[39] Hiskey,[40] and Chance.[41]

Techniques of Difference Spectroscopy

Dual or Split Beam Spectrometry

Difference spectroscopy is usually performed in a double beam instrument where light passes either simultaneously or alternately through the sample and reference solution. There are several methods commonly used for measuring difference spectra. For example, Herskovits and Lascowski[42] have designed a tandem cylindrical cell. One tandem cell is placed in the sample beam containing protein plus perturbant (or ligand) in one compartment and water in the other. In the reference beam water plus perturbant is placed in one compartment and protein in the other. The contents of two compartments of both cells may be mixed to obtain the baseline. Yankeelov[43] has designed a split compartment cell with a chamber for mixing on the top of the cuvette. In his technique the sample is placed in one compartment and the perturbant is placed in the other compartment of both the sample and reference cuvettes, and a baseline is read. After the baseline is recorded the contents of the sample cuvette are mixed. The cell designed by Yankeelov has the advantage of fitting into a standard 1-cm cuvette cell holder, and the samples can be mixed without the

necessity of transferring them to a separate mixing compartment. In addition, various authors have used two standard cuvettes in tandem in each beam where the contents of the sample compartment cuvettes are mixed to generate the difference spectrum.[44-46]

Dual Wavelength Spectrophotometry

Dual wavelength spectrophotometry is often used to measure the small absorbance changes superimposed on a large backbone. Several dual wavelength spectrophotometers are commercially available.[47] In dual wavelength spectrophotometry the spectrometer measures the difference in absorption between two different wavelengths in the sample cuvette, A (λ_1 λ_2). If one chooses wavelengths carefully, it is possible to find wavelength pairs where the absorbances of the ligand or the protein are equal. The difference in absorbance between λ_1 and λ_2 will then be 0. Thus, after the dilution correction, any changes in absorbance recorded when ligand is added to enzyme (or vice versa) will be directly proportional to the amount of enzyme-ligand complex formed:

$$\Delta A(\lambda_1 \lambda_2) = \epsilon_1[PX] + \epsilon_2[PX_2] \cdots + \epsilon_n[PX_n] \quad (33)$$

where

$\epsilon_1, \epsilon_2 \ldots \epsilon_n$ = the extinction coefficients for the changes in absorption;

$[PX], [PX] \ldots [PX_n]$ = macroscopic concentrations of the various complexes formed.

The technique of dual wavelength spectroscopy thus allows one to measure "difference" spectra directly. It has the advantage that spectral titrations can be done in one cuvette. Errors due to the incorrect matching of volumes in small additions to sample and reference cuvettes and errors due to incomplete mixing of two samples are eliminated. The method of dual wavelength difference spectral titration was pioneered by Theorell and Chance.[48] If it is difficult to pick wavelengths where the contributions of ligand are equal and cancel, it is, of course, possible to do a titration by correcting for the contribution of free ligand.

Another application of dual wavelength spectrophometers is the determination of the first derivative of an absorption spectrum. In this technique the difference in absorption between two wavelengths at a fixed separation (e.g., 5 Å) will be taken as a function of wavelength. This technique sharpens the structure of absorption bands and may occassionally be of use in identifying particular chromophores.[32,49] Note that Brandts and Kaplan[49] have recently had instrumental difficulties with this technique, as their data obtained automatically did not agree with the same data taken manually. The cause of this discrepancy was not explained by the authors, but one should bear in mind the possibility of artifacts when performing instrumental measurements.

Low Temperature Spectroscopy

Another technique of sharpening the fine structure of the absorbance bands due to the aromatic chromophores of proteins is to cool the proteins to 77° K. This technique has been pioneered by Strickland and co-workers, who have used the technique to resolve bands due to tryptophan in the spectrum of peroxidases and cytochrome C.[50]

Applications of Difference Spectroscopy

Determination of Binding Constants

The general theory of obtaining binding constants from difference spectral measurements has been discussed in the first section of this paper. (For a review discussing some of the first studies of difference spectroscopy of enzyme-coenzyme complexes, see Shifrin and Kaplan.[51]) For example, horse liver alcohol dehydrogenase forms a complex with DPNH which is characterized by a shift in the absorption maximum of the reduced coenzyme from 340 to 325 nm. This property has allowed the determination of the binding constant.[48,52]

The Effects of Ligands on the Intrinsic Spectra of Proteins

Solvent Perturbation

Among the most frequent uses of difference spectroscopy is the monitoring of the perturbation of the chromophores of a protein by solvents.[53] Often, this spectral perturbation is due to a wavelength shift. To a first approximation the difference spectrum of perturbed protein vs unperturbed material resembles the first derivative of the protein absorption spectrum. The perturba-

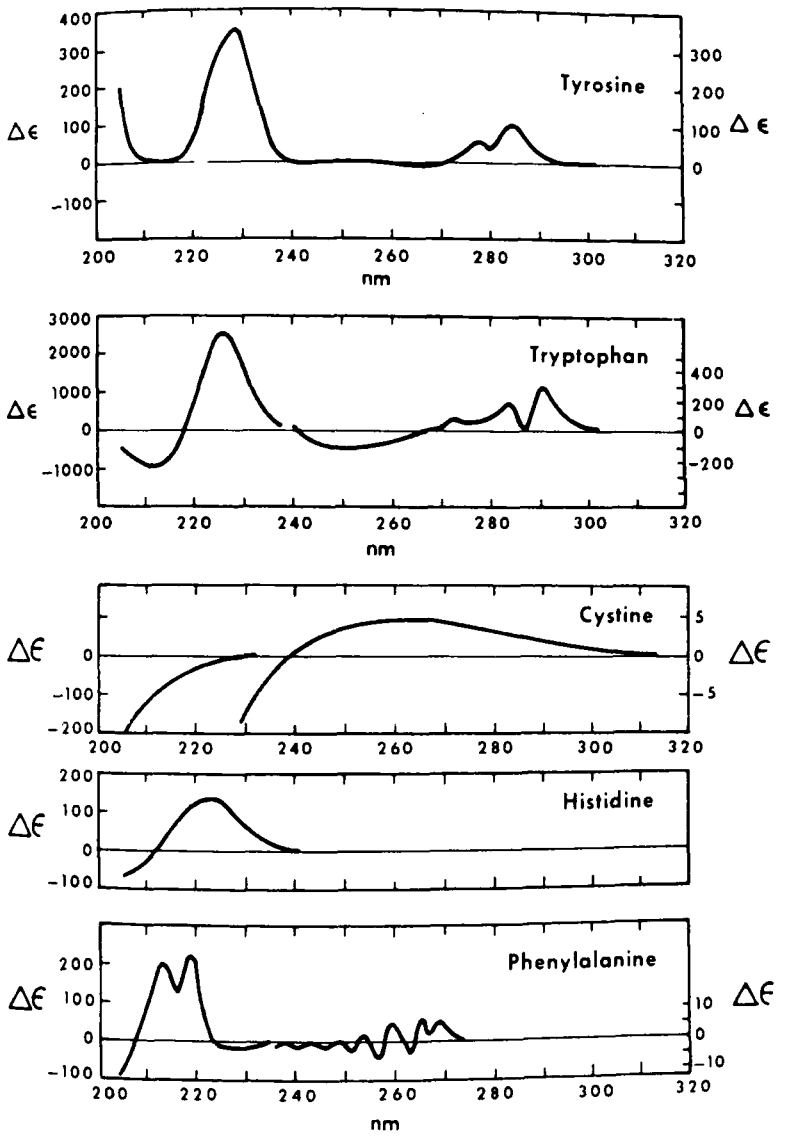


FIGURE 5. Perturbation of aromatic chromophores by 20% ethylene glycol. (From Donovan, J. W., *J. Biol. Chem.*, 244, 1961, 1969. With permission.)

tion of proteins has been reviewed.[36-38] The change in the spectrum of a protein caused by changing solvents may be caused by direct interaction of the chromophore with a perturbing solvent or may be an inductive effect due to a change in charge somehow relayed to the chromophore. Donovan[37,38] discusses some of the mechanisms which may affect the absorbance of a chromophore. Herskowitz and Lascowski[42, 53] developed the technique of using difference spectra to determine which of a protein's chromophores are exposed to solvent. While not actually concerned with enzyme-ligand interactions, their papers are interesting because they illustrate the effect of changing the solvent polarity on the spectra of proteins; the same types of changes are often observed when ligands bind to proteins.

Bailey et al.[44] have analyzed the perturbations in the UV absorption spectra of proteins and model compounds caused by addition of the lower polarity. For both tyrosine and tryptophan, addition of the lower polarity solvents leads to red shifts and changes in peak intensity and oscillator strengths. Similarly, Donovan[54] has obtained the perturbation difference spectrum of various aromatic amino acids caused by 20% ethylene glycol. His spectra, shown for reference, are portrayed in Figure 5.

The spectra obtained from solvent perturbation

techniques have been very useful in the interpretation of UV difference spectra due to enzyme-ligand complex formation. For example, Matthews et al.[55] have studied the UV difference spectra of the lactose repressor in the presence of inducers and anti-inducers. They also studied the effect of solvent perturbation on the protein in the presence of the same inducers and anti-inducers. It appears that the inducers cause changes in the environment of multiple aromatic regions, while the anti-inducers have no effect on the UV spectrum of the aromatic residues. Moreover, fewer aromatic residues are available to solvent in the presence of inducers than in the presence of anti-inducers. The authors suggest that the binding of the inducers causes a conformational change, while the binding of the anti-inducer does not.

Kayne and Price[56] used difference spectroscopy as part of a study to monitor conformational changes in the allosteric inhibition of muscle pyruvate kinase. Phenylalanine addition to the enzyme produces alterations in the UV difference spectrum, suggesting a change in the environment of tryptophan residues. Mg^{++} addition decreases the binding constant of the phenylalanine and serves to lower the difference spectrum which is restored by higher concentrations of phenylalanine.

In a recent study Crowder et al.[57] have performed UV difference spectroscopic studies of the binding of ligands to rabbit muscle aldolase. Binding of D-arabinitol-1,5-diphosphate, an inhibitor of the enzyme, causes a change in the spectrum of the tryptophan residues. There is a sharp minimum at 298 nm which is probably due to a blue shift of the spectrum of a tryptophyl residue exposed to a more polar environment. In contrast, 1,5-pentadiol-diphosphate does not generate this difference spectrum, suggesting that the hydroxyl residues of the D-arabinitol-1,5-diphosphate are necessary to produce the change in the difference spectrum. The effect of the abrabinitol derivative is lessened in the presence of ethylene glycol, suggesting that the ligand binding increases the exposure of tryptophan to the solvent.

Fisher[58] has recently reviewed the spectral studies of ligand binding to glutamic dehydrogenase. Using the solvent perturbation technique, he has assigned different aspects of the difference spectrum to interactions involving the rings of NAD(P)H and those of the tryptophanyl and tyrosyl residues on the surface of the enzyme. The study is an excellent example of the use of difference spectroscopy to measure enzyme-ligand interaction.

Spectral Titrations

UV difference spectroscopy may also be useful in following the pH titration of proteins. For example, the phenol group of tyrosine has an absorption band at 295 nm for the ionized form, which is lacking in the unionized form; thus, the UV spectrum can be used to monitor the ionization of tyrosine. Moreover, the pH titration of a residue that is not itself a chromophore may be mirrored in the UV spectrum of another residue which is a chromophore, and spectral titrations may give general information about protein side chain interactions. The subject of spectrophotometric titration of proteins has most recently been reviewed by Donovan.[59]

An interesting study of enzyme-substrate interaction utilizing spectrophotometric titrations was performed by Iida and Ooi[60] on the titration of ribonuclease T_1 in the presence and absence of the inhibitor 2′-guanosine monophosphate. As part of a potentiometric and spectrophotometric study of the enzyme, the authors monitored the change in absorbance at 295 nm as a function of pH. The absorbance increases only slowly with pH until pH 10.5, at which point it increases sharply. At pH values greater than 10.5 the absorbance change is time dependent. The titration is reversible below pH 10.5. The data show that most of the tyrosines are buried within the molecule and that at most two tyrosines have normal reversible ionizations. When the inhibitor is added the break in the titration curve increases to pH 10.8; moreover, one of the accessible tyrosines becomes masked.

Another interesting use of spectral titrations has been made by Mulvey et al.[61] as part of a study contrasting the spectral properties of human and hen egg white lysozyme. Hen egg white lysozyme contains six tryptophans and three tyrosines. Of these residues, three tryptophans (62, 63, and 108) appear to be in the binding cleft, and they all interact with a saccharide ring. Human lysozyme has five tryptophans and six tyrosines. One of the tyrosines (63) corresponds to tryptophan 62 of hen egg white lysozyme; two tryptophans (64 and 109) are homologous to tryptophan 63 and 108. The spectrophotometric difference spectra of the enzymes from both sources were

performed with a series of saccharides from di- to penta-*N*-acetyl-D-glucosamine. For hen egg white lysozyme the spectra are pH independent and have features typical of tryptophan perturbation spectra. The analysis of human lysozyme is similar, but the spectra, in addition, show positive peaks at 250 and 3000 nm associated with tyrosine. These peaks grow as the pH increases to 10 and then diminish. When the neutral difference spectrum is subtracted from the alkaline spectrum, the spectrum resembles the ionization difference spectrum of tyrosine. The two maxima, however, are at longer wavelengths than the spectrum of acetyltyrosine in aqueous solution. Using spectral maxima at 245 and 290.5 nm, the authors[61] followed the titration of human lysozyme and the inhibitor complexes and reached the conclusion that the binding of inhibitor lowers the pK of a tyrosine from approximately 10.5 to 10.0. The authors suggest that this tyrosine is the tyrosine 63 found in the binding site.

The Effect of Binding on the Absorption Spectrum of the Ligand

The State of Ionization of the Ligand

If the state of ionization of a ligand which is a chromophore shifts when the ligand binds to an enzyme, large easily interpreted spectral changes may occur. There are some very interesting studies which have utilized these changes to monitor enzyme-ligand interactions.

For example, Deavin et al.[62] have studied the difference spectral perturbations of bovine pancreatic ribonuclease upon interaction with nucleotides. For guanine nucleotides these changes are the same as those produced by protonation of the guanine chromophore. It is concluded that the guanine ring of nucleotides interacts with an acid group of the protein in position N-7. The enzyme seems to have a similar interaction with adenine nucleotides. For uridine nucleotides the spectral changes on interaction with the enzyme could be mimicked by deprotonation of the pyrimidine ring in a partially hydrophobic environment. On the other hand, the spectral changes for cytidine nucleotides could not be reproduced by pH or other chemical modifications. The triphosphate of uridine, cytidine, guanine, and adenine all form 1:1 ligand-enzyme complexes.

Walz and Hooverman have similarly studied the interaction of guanine ligands with ribonuclease T_1.[63] They also discovered that for a series of different guanine derivatives the difference spectra resembled an acid induced perturbation; however, there are some differences, suggesting that other unknown factors entered into the difference spectrum.

Recently, the interaction of dihydrofolate reductase with substrates and inhibitors has been studied by various groups.[64-66] Erickson and Mathews[64] studied the spectral changes associated with the binding of aminopterin, methotrexate, and N^{10} formyl-aminopterin to T_4 dihydrofolate reductase and found that the spectrum was similar to the difference spectrum obtained for the free compounds in acidic vs neutral solutions, suggesting that the 4-amino-folate compound bind in the cationic form. Folic acid, dihydrofolate, and dihydroaminopterin, however, did not show similar changes. The cofactor NADPH did not cause a difference spectrum when added alone to the reductase from T_4, nor did it perturb the spectrum of the complex with aminopterin. It did, however, perturb the spectrum of the complex with dihydro-aminopterin. The authors suggest that the binding site of the reduced inhibitor might not be the same as that of aminopterin itself.

Poe et al.[65,66] independently studied the binding of inhibitors and substrates to dihydrofolate reductase from *E. coli*. The binding of methotrexate to the enzyme from *E. coli* generates a difference spectrum almost identical to the difference spectrum seen with T_4, suggesting that the inhibitor binds near negatively charged groups on the enzyme which stabilizes the protonated form of the inhibitor. Folate and dihydrofolate show smaller difference spectra which are difficult to interpret and do not resemble the spectra of the T_4 complexes. Moreover, the binding of NADPH to the enzyme produces a difference spectrum not seen with the T_4 enzyme, and NADPH also alters the difference spectrum of the methotrexate-enzyme complex. The two studies thus illustrate the similarity of the factors determining the difference spectrum of methotrexate on the surface of dihydrofolate reductases from two sources which have quite dissimilar physical properties.

Ligand Environment

The placing of a ligand from a polar to a nonpolar environment on the enzyme surface may cause spectral shifts that may be used to interpret which part of a ligand interacts with the enzyme.

For example, Fisher[58] has reviewed the factors which influence the binding of NADPH to glutamate dehydrogenase. The difference spectrum contains contributions from three sources: (1) a red shift and hypochromicity of the 340-nm band of reduced nicotinamide; (2) a hypochromicity without any shifts of the adenine band; (3) perturbation of aromatic chromophores. Fisher has interpreted these bands in the following way. The reduced nicotinamide ring is interacting directly with the enzyme surface, while the adenine group is not in contact with the surface but is more or less coplaner with some chromophore of the complex. The spectrum of NADH, in addition to the bands of NADPH, shows a shifted adenine component with a different concentration dependence than that of the rest of the complex, showing an additional mode of binding. Fisher and coworkers used studies similar to these to map out a mechanism of glutamate dehydrogenase.

Charge Transfer Interactions

In addition to spectral perturbations of the ligand and protein chromophores, protein-ligand interactions may result in the formation of new bands due neither to the protein nor the ligand. These bands are commonly called charge-transfer bands, although they do not necessarily obey the criteria of a true "charge-transfer" complex. (For a definition of charge-transfer complexes, see Jencks.)[67] For example, Racker and Krimsky[68] noted that when NAD^+ binds to glyceraldehyde-3-phosphate a new band appears at 360 nm associated with complex formation. Charge-transfer bands may be of use in monitoring enzyme-ligand interactions and may help interpret the forces responsible for enzyme-ligand binding. Charge-transfer complexes are also sometimes used as a probe of enzyme structure.

For an example of the latter use, Deranleau et al.[69] have used *N*-methylnicotinamide chloride as a conformational probe of chicken egg white lysozyme. Hen egg white lysozyme forms a yellow complex with *N*-methylnicotinamide chloride. Titrations studied indicate that the weak complex has an association constant of 3.2 liter mol^{-1}, and that there is only one single class of binding site on the lysozyme molecule. Comparison with model systems suggests that the complex is a charge-transfer interaction with indole as the donor and the nicotinamide derivative as the acceptor. The geometry for such a complex requires that the ring faces of both donor and acceptor be completely available for complexation, suggesting that lysozyme has at least one tryptophan indole ring-face freely available to the solvent. Robbins and Holmes[70] performed a similar study on lactalbumin and found that at pH 6 one tryptophan residue is exposed and complexes, while two are exposed at acid pH, and three of the four tryptophans complex at pH 11.

CIRCULAR DICHROISM AND OPTICAL ROTATORY DISPERSION

Considerable information about the effects of ligands upon proteins can be obtained from measurements of the optical activity of the protein in the presence and absence of the ligand. Two forms of optical activity commonly measured are optical rotatory dispersion (ORD) and circular dichroism (CD). ORD is the measurement of a molecule's ability to rotate the plane of linearly polarized light as a function of wavelength. CD is the measurement of the molecule's unequal absorption of right-handed and left-handed circularly polarized light. Both ORD and CD are properties of asymmetric molecules, and the two phenomena have the same molecular origins. The ORD, due to any particular electronic transition, tails out over the entire optical spectrum, while circular dichroism is found only in the region of the absorption band of a particular transition. The ORD and CD of a typical absorption band are shown in Figure 6.

The use of optical activity to measure protein conformation has been well-documented and there are several excellent reviews.[71-88] What is sometimes overlooked is that ORD and CD are very sensitive spectral tools and, thus, may be of use in studying many protein-small molecule interactions which cause changes in optical activity. (1) They can be used to detect the effect of a ligand on protein backbone conformation; (2) The aromatic amino acids are optically active and, occasionally, proteins show ORD and CD bands which can be assigned to specific amino acids; thus, ORD and CD may be used to study the effects of ligands upon specific amino acid residues; (3) Often, the interaction of a ligand, which is a chromophore, but is itself optically inactive, will induce large "extrinsic" CD and ORD bands when bound to proteins. These bands are useful in following enzyme ligand binding; (4) Occasionally, the optical activity

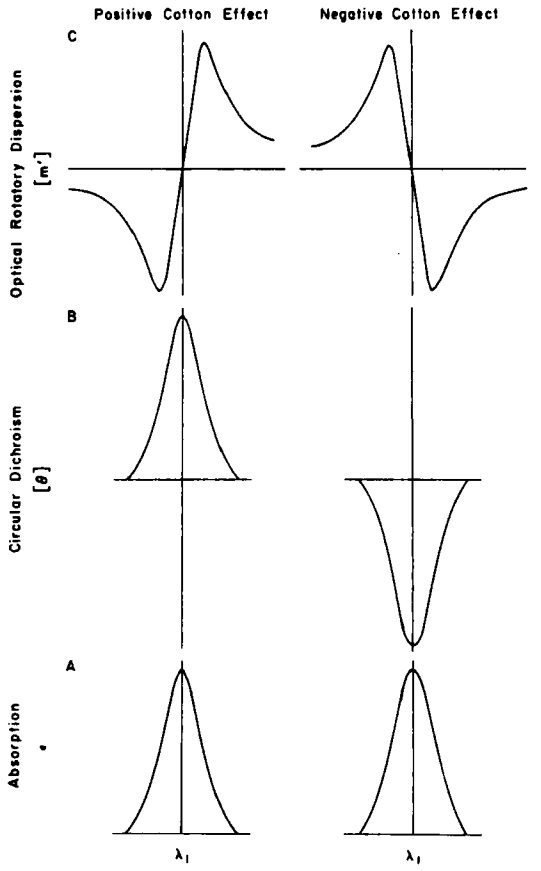


FIGURE 6. A typical extrinsic absorption band (A) with its associated circular dichroism (B) and optical rotary dispersion (C) curves. (From Adler, A. J., Greenfield, N. J., and Fasman, G. D., *Methods in Enzymol.*, 27(D), 675, 1973. With permission.)

induced in the spectrum of the ligand when it binds to a protein can give information about the nature of the ligand on the protein's surface. For example, it may help describe the state of ligation of a metal ion or help decide which conformer of a disymmetric chromophore binds to a protein.

The Effects of Ligands on Protein Structure

A complete review of the various methods for determining protein structure from ORD and CD is beyond the scope of this paper as there are many excellent reviews in the literature.[71-88] To provide a brief background, in 1955 Cohen[89] suggested that the change in optical rotation that was noted upon protein denaturation may be due to a helix to random-coil conformational transition. Since that time several empirical equations were found to correlate ORD and CD with polypeptide structure. The first measurements were made in the visible and near UV. Several equations were found to correlate the alpha-helix with optical rotation. These include the Drude equation,[90]

$$[\alpha]_\lambda = \frac{A}{\lambda^2 - \lambda_c^2} \tag{34}$$

where

$[\alpha]_\lambda$ = Specific rotation at each wavelength λ;
A = constant;
λ_c = constant.

Yang and Doty[91] found that for polypeptides with low helical content, λ_c was proportional to helicity up to about 40%.

Moffitt[92-94] developed an equation which described the rotation of a helical polypeptide in the visible region

$$[m'] = \frac{3}{n^2 + 2} \frac{M_0[\alpha]}{100} \tag{35}$$

$$= \frac{a_0\lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

where

$[m']$ = the reduced mean residue rotation;
$3/(n^2 + 2)$ = correction for the refractive index (n) of the medium;
M_0 = mean residue weight;
$[\alpha]$ = specific rotation;
λ_0 = average absorption maximum for protein;
λ = wavelength of the rotation;
b_0 = constant;
a_0 = constant.

Principally, b_0 is a function of the helical backbone. The plotting of $m'(\lambda^2 - \lambda_0^2)$ against $1/(\lambda^2 - \lambda_0^2)$ gives a straight line when λ_0^2 is chosen correctly. λ_0 =212 is most widely accepted. It was found experimentally[93] that the ORD of many fully helical polypeptides gave a value of b_0 = -630, whereas fully random polypeptides gave a value of b_0 = 0. Polypeptides of mixed conformation gave a b_0 value proportional to helicity; thus, the value of b_0 was used as a measure of helical content.

In the region of the absorption spectrum, an optically active molecule may have a large

anomalous rotation called a Cotton effect. In 1960 Simmons and Blout[95] were the first to obtain ORD measurements in the absorption region of the polypeptide backbone. They detected the trough of a Cotton effect at 233 nm for the tobacco mosaic virus. Following this, instrumentation improved rapidly, and measurements on the entire ORD and CD spectra of proteins and polypeptides from 190 to 300 nm were achieved. The α-helix has an ORD trough at 233 nm and a CD negative maxima at 222 nm; thus, measurements of the ORD[96] and CD[97,98] at those two wavelengths were used to estimate helical content.

In 1966 good ORD and CD measurements of the β form of poly-L-lysine in solution were obtained.[99-101] Once this reference spectrum for β structure was obtained, it was attempted to fit the ORD and CD of proteins by a linear combination of the curves for polypeptides in the α-helical, β, and random conformations.[102-104] Recently much work has been done in refining the reference spectra by examining the CD and ORD of proteins whose conformation is known from X-ray crystallography and making the assumption that the spectral data of many proteins can be fit by the same three reference spectra.[105-110] The ORD and CD of poly-L-lysine in the α-helix, β, and random structure are shown in Figures 7 and 8.

As techniques for monitoring changes in the peptide backbone of a protein from its ORD and CD spectra have been developed, they have been used to detect backbone changes when ligands bind to proteins. The measurements are difficult because often the ligands will themselves be chromophores which may become optically active when bound to the protein. Several attempts have been made, however, to determine conformational changes from changes in the parameters associated with the peptide backbone.

The earliest work used the Moffit parameters for examining protein structure.[71-88] For example, Rosenberg et al.[111] studied the ORD of liver ADH in the presence of its coenzymes and some inhibitors. They tried to correct for the contribution of bound NAD + and NADH to the Moffit equation and concluded that binding of the coenzymes was accompanied by conformational changes. Similarly, Bayley and Radda[112] found that NADH and GTP had an effect on the Moffit parameters of the ORD of glutamate dehydrogenase. They suggested that NADH and glutamate

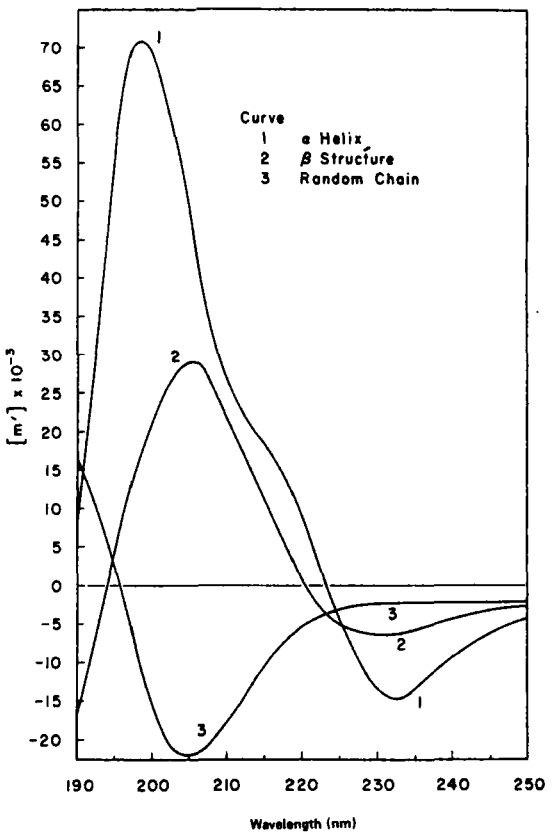


FIGURE 7. The optical rotary dispersion of poly-L-lysine in the α-helical, B, and random conformation. (From Greenfield, N. J., Davidson, B., and Fasman, G. D., *Biochem.*, 6, 1630, 1967. With permission.)

in the presence of NADH causes conformational changes when bound to the enzyme.

The ORD of the Cotton effect of the peptide backbone was utilized as instrumentation became available. Dratz and Calvin[113] studied the interactions of substrates and inhibitors on aspartate transcarbamylase by monitoring the ORD at 233 nm. Upon addition of CTP, α_{233} increased; upon addition of succinate, α_{233} decreased sigmoidly. The authors suggest that conformational changes were responsible for the effect. Likewise, Jaenicke and Gratzer[114] studied the binding of cofactors to yeast glyceraldehyde-3-phosphate-dehydrogenase by monitoring the ORD trough at 233 nm.

More recently, CD measurements have been increasingly employed. For example, CD measurements have been used by Ohta et al.[115] to measure the interactions of tyrosine RNA synthetase with its specific transfer RNA, and they have suggested that the interaction decreases the helical content of the enzyme.

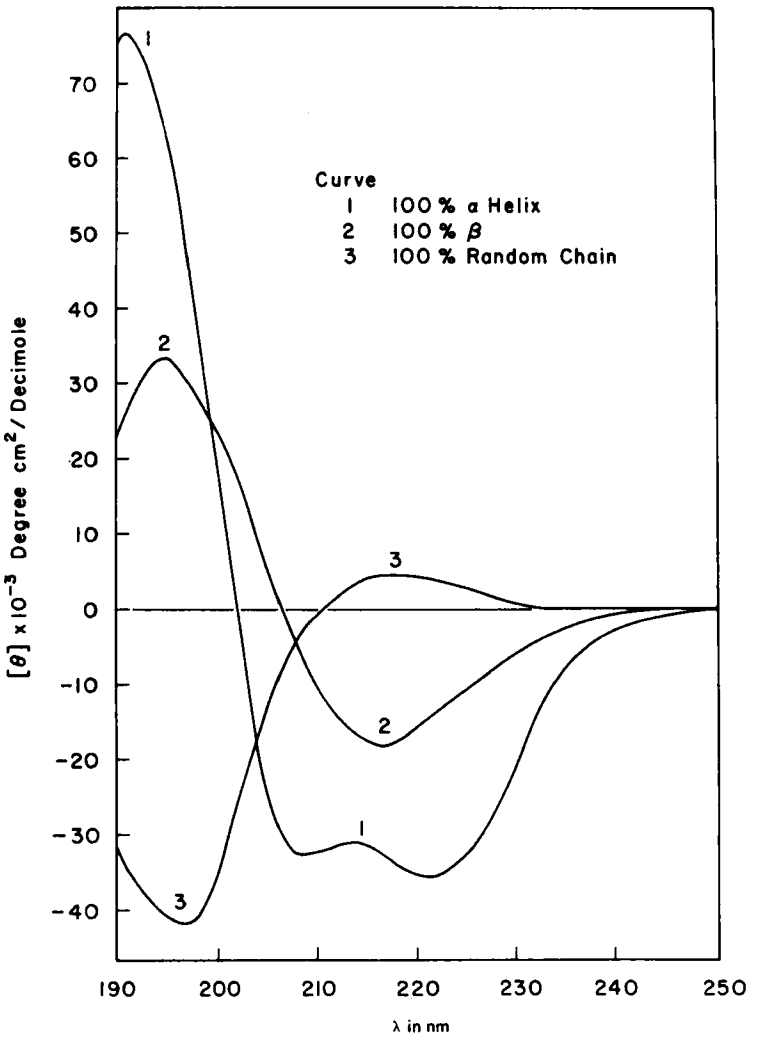


FIGURE 8. Circular dichroism spectra of poly-L-lysine in the α-helical, B, and random coil conformation. (From Greenfield, N. J. and Fasman, G. D., *Biochem.*, 8, 4108, 1969. With permission.)

In a recent example McCubbin and Kay[116] have used CD to measure the interaction of calcium ion with troponin A. There is a marked increase in the magnitude of the elipticity of the 221-nm CD band, suggesting an increase in helical content upon calcium addition. When troponin is modified by replacing a varying number of carboxyl groups by glycinate residues, the samples are only slightly affected by Ca^{++} ions. The authors conclude that the effect of calcium upon troponin is a neutralization of charges on aspartic acid and glutamic acid residues which results in a conformational change.

In addition to the study of specific enzyme-ligand interactions, the ORD and CD of proteins have been used to monitor various interactions of proteins with denaturing agents. These studies have been extensively reviewed and are beyond the scope of this paper.[71-88]

The Effect of Ligands on the Intrinsic CD Bands of the Aromatic Amino Acids in Proteins

As the technology for measuring CD and ORD has improved, the factors influencing the optical activity of enzyme-ligand complexes have been better analyzed. Circular dichroism has the potential for delivering a great deal of specific information about the aromatic amino acid residues involved in ligand binding, although great care must be taken in analyzing data.

For example, in an early study Omenn et al.[117] tried to gain specific information on the role of

aromatic residues from a study of the CD of staphylococcal nuclease. This enzyme contains one tryptophan and seven tyrosine residues. The CD shows a small positive band at 296 nm, which the authors have attributed to the tryptophan, and a larger negative band at 277 nm, which they have assigned to the tyrosyl residues. Upon binding of the inhibitor, deoxythymidine 3′5′-diphosphate, in the presence of Ca^{++} there is a large change in the CD spectrum. The authors suggest that the CD changes are due to changes in the asymmetric environment of tyrosyl residues upon nucleotide binding. The results, however, are complicated by the overlap of the spectrum of the inhibitor with the spectrum of the enzyme.

Considerable work has been done on the CD of lysozyme because the structure is known from X-ray crystallography and, thus, it is possible to correlate the optical properties with the structure of the enzyme. Moreover, the spectra of the saccharide substrates and inhibitors do not contribute to the near UV absorption. Among the first to study the effect of inhibitor binding to lysozyme using CD were Glazer and Simmons.[118] When *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine bind to lysozyme, the CD spectrum is perturbed. The positive bands at 292, 287, and 282 nm increase, but a negative band at 262 nm is unchanged. Ethylene glycol has an effect similar to the substrate, while treatment with sodium dodecyl sulfate removes the bands. The authors suggest that the 262-nm band is due to disulfide bonds, and the high wavelength bands are due to aromatic chromophores. They conclude that the inhibitors displace water from these residues at the binding site of the protein.

Teichberg et al.[119] tried to more definitively assign some of the CD bands of lysozyme. They attempted to demonstrate the contribution of tryptophan 108 by comparing the CD spectrum of native lysozyme with lysozyme that had been selectively oxidized with iodine. They also tried to separate out the contributions of the tyrosine residues by comparing the spectrum of lysozyme at neutral pH with the spectrum of lysozyme at pH 11 (where the tyrosines would be ionized). *N*-Acetyl-D-glucosamine perturbed the spectrum of the unmodified enzyme but had no effect on the spectrum of the oxidized enzyme. The authors concluded that the main effect of the CD was a result of a change in orientation of tryptophan 108 when the inhibitor bound to the enzyme. Teichberg et al., however, assumed that the iodine oxidation formed the oxindole derivative of tryptophan 108. Imoto et al.[120] have pointed out that iodine oxidation results in the formation of an ester between glutamate 35 and tryptophan 108. The formation of this ester results in the formation of a new asymmetric center and is responsible for the CD changes noted by Teichberg et al.[119] When the true oxidole derivative is actually prepared, it is shown that tryptophan 108 makes negligible contribution to the spectrum of native lysozyme. Imoto et al.[120] suggest that the changes observed in the CD upon saccharide binding are due instead to perturbations of tryptophan 62.

On the other hand, CD measurements often give unambiguous results. For example, Ikeda and co-workers[121-124] have reported detailed studies of lysozyme spectra over the pH range 7 to 12.8. They have been able to use the CD spectra to distinguish three tyrosine ionizations. They also used the increase in the CD band resulting from *N*-acetyl-glucosamine binding to study the saccharide interaction.

The ionization of tyrosine has also been of use in assigning the CD spectrum of ribonuclease.[125-129] The CD spectrum has been shown to contain a positive extremum near 241 nm which is highly sensitive to the environment of a tyrosyl residue.[128] This extremum is near 228 nm for normal tyrosine groups, but near 245 nm for the ionized form in alkaline solutions. In addition, this extremum has been shown to appear near 241 nm for certain uncharged but perturbed tyrosyl residues buried near charged side chains in hydrophobic environments. Simons[129] utilized the 241-nm band of ribonuclease to follow its titration with the inhibitor 3′-CMP (cytidine monophosphate). The addition of 3′-CMP to ribonuclease apparently was accompanied by the increased accessibility to solvent of a previously buried tyrosine.

Recently Strickland's laboratory[130-132] has been able to differentiate the CD bands arising from various side chain residues by examining their vibronic fine structure. Much of this has been performed at low temperature where band resolution is better. This technique is potentially of great use. For example, Fretto and Strickland[133] have utilized aromatic fine structure to examine the interaction of substrates and inhibitors with carboxypeptidase A. The addition of β-phenylpropio-

nate to carboxypeptidase A makes the CD more positive in the region from 270 to 285 nm, apparently resulting from interactions with tyrosine residues. From comparisons with crystallographic[134] and kinetic[135] data the authors assigned the band to tyrosine198. Glycyl-L-phenylalanine does not produce any major alterations in the tyrosyl CD bands, although crystallographic data show movement of tyrosine 248 when the inhibitor binds. The binding of glycyl-L-phenylalanine, glycyl-D-phenylalanine, and L- and D-phenylalanine shifted the wavelength positions of the tryptophyl CD fine structure. The author's suggest this effect is a result of binding outside of the active site pocket.

The Induction of Extrinsic Cotton Effects by the Binding of Ligands to Proteins

The effects of ligands on the intrinsic ORD and CD of proteins are interesting but are often hard to interpret when the ligands themselves are chromophores. These chromophores may induce a change in the ORD and CD which mimics a change in the conformation of the protein which actually is a pure optical effect.

Of potentially greater value is that when chromophoric ligands bind to a protein they are often bound in an asymmetric fashion. This binding may induce the bands of the ligand to become optically active or may increase the optical activity of an already optically active ligand. These changes in optical activity may be used to measure ligand binding to the protein and may often give insight into the environment of the ligand or the conformation of the ligand when bound to the protein. The generation and usefulness of these "extrinsic" Cotton effects has been reviewed by Ulmer and Vallee[136] and Perrin and Hart.[137]

Among the first uses of the "extrinsic" Cotton effects was the demonstration of the formation of enzyme-ligand complexes and the determination of binding constants. For example, Ulmer, Li, and Vallee[138-140] utilized optical rotatory dispersion to measure the binding of substrates and inhibitors to alcohol dehydrogenase. Simpson and Vallee[141] showed that the ORD of FAD when bound to diaphorase was ten times greater and opposite in sign than when the coenzyme was free in solution. Takagi et al.[142] found similar results when they monitored the binding of FAD to D-amino acid oxidase by CD. Brady and Beychok[143] likewise utilized CD to monitor the binding of FAD to lipoyl dehydrogenase; Koberstein and Sund[144,145] and Jallon and Iwatsubo[146] were able to show that there were two DPNH binding sites on glutamic dehydrogenase using CD. CD has also been used to measure nonspecific enzyme-ligand interactions. For example, Sjöholm and Grahnén[147] were able to demonstrate that human serum albumin has at least two types of binding sites for tryptophan by examining the extrinsic circular dichroism bands generated by the binding of the amino acid.

A potentially useful technique for measuring extrinsic Cotton effects due to protein-ligand interaction is the method of difference spectropolarimetry developed by Adkins and Yang.[148] They have modified a single-beamed spectropolarimeter to function as a double-beamed instrument by reflection of its beam through reference solutions whose rotatory contributions are then optically subtracted from the sample solution in the incident beam. They have used this technique to measure the binding constant of *N*-acetyl-D-glucosamine to lysozyme.

Protein Metal Interactions

It was first observed by Lifshitz[149] that the absorption bands of metals become optically active in the presence of an inner sphere asymmetric ligand. Peptides and proteins thus often form asymmetric complexes with metals which give rise to Cotton effects. These effects may give insight into the environment and state of ligation of metals in metalloproteins and may also be used as biological probes in proteins which do not require metals for activity, but which form metal-protein complexes. In the latter case the metals may stimulate or inhibit protein function.

The field of metalloprotein chemistry is very large; thus, the study of the optical activity of metal-protein complexes encompasses many fields. The optical activity of metals has been studied in heme proteins such as hemoglobin, myoglobin, and cytochrome;[150-156] nonheme iron-containing proteins such as ferredoxin,[157] hemerythrin,[158] and metapyrocatechase;[159] copper-containing proteins such as azurin and laccase,[160] hemocyanin,[161] and galactose oxidase;[162,163] and concanavalin A which can utilize several transition metals.[164]

In addition to the study of metal-containing proteins which are naturally optically active, it is

possible to induce optical activity in proteins by the substitution of colored metal ions for transparent ones. There is a wide range of studies where cobalt has been substituted for zinc.[165-167] These studies include work on carbonic anhydrase[166] and lipoamide dehydrogenase.[167] Copper has also been substituted as a probe of the iron binding site in transferrin,[168] and Cadmium has been substituted for zinc as a probe of aspartate transcarbamylase.[169] Metals have been used as probes of enzymes which have no functional metals. These studies include the binding of copper to myoglobin[170] and ribonuclease,[171] and the binding of various metal probes to lysozyme.[172]

Obviously, a complete discussion of the large literature on metal-protein interactions would be a review in itself. Thus, only some papers will be discussed that illustrate some of the uses of the optical activity of metal-protein complexes. (For a review of some of the earlier work, see Ulmer and Vallee[136] and Perrin and Hart.[137])

An interesting paper by Kalb and Pecht[164] utilizes the circular dichroism of metal ions required for protein activity in a study of the cobalt complexes of concanavalin A. Concanavalin A is a saccharide-binding protein composed of two identical subunits. Each molecule has two equivalent saccharide binding sites which require metal ions. Demetallized protein binds one transition metal ion per subunit. Binding of transition metal ions brings about the formation of one Ca^{+2} binding site per subunit. Both metals are needed for saccharide binding. The CD of Co^{++} concanavalin A consists of at least four positive transitions between 400 and 600 nm with high optical anisotropy. When Ca^{++} is bound, two of the CD transitions disappear. No further changes occur on binding of α-methyl glucoside. In contrast, Ca addition has no effect on the visible absorption spectrum of Co^{++} concanavalin A. The authors suggest that the transition metal binding site has nearly octahedral symmetry with some deviation from centrosymmetry. The deviation is decreased on binding of Ca^{++} to the protein, resulting in the loss of two of the CD bands.

Two interesting papers also illustrating the use of the intrinsic CD bands of metal-containing proteins on the CD and optical spectra of galactose oxidase are by Ettinger[162] and Ettinger and Kosman.[163] Galactose oxidase is a protein which contains a single cupric copper atom per molecule. The protein has four CD bands between 300 and 630 nm and an additional band near 775 nm. Since more than four maximally allowed d-d transitions are observed, Ettinger suggests that there is both ligand orbital and charge transfer character to one or more of the detected transitions.[162] In the absence of oxygen D-galactose causes a marked reduction of the CD bands due to copper. Oxygen, however, has almost no effect on the copper CD bands by itself. The authors conclude that during the course of the enzyme reaction, oxygen binds to the enzyme after galactose and interacts directly with the substrate rather than with the inner coordination sphere of the copper atom.[163]

A recent example of a paper utilizing substitutions of one metal for another is the work of Griffen et al.[169] on aspartate transcarbamylase. Replacement of Zn^{++} by Cd^{++} gives rise to an intense CD difference spectrum. The CD spectrum due to cadmium has been used to assess the effect of substrates and regulators on the metal ion which is essential for the association of the isolated subunits into the native enzyme. The binding of carbamyl phosphate and succinate perturbs the band due to cadmium, while the feedback inhibitor, CTP, does not. To monitor the linkage between conformational changes at different loci in aspartate transcarbamylase, CD changes at different wavelengths were followed during ligand titrations. The carbamyl phosphate and succinate concentrations needed for half maximal changes in the ellipticity as a function of substrate concentration was not the same, depending on the wavelengths examined. The authors suggest that the results demonstrate the existence of multiple conformational equilibria for the binding of these substrate ligands and that allosteric interactions of aspartate transcarbamylase cannot adequately be described by a two-state model.

Another interesting paper illustrates the use of CD to study ligand binding to a protein which does not require metals. Nickerson and Van Holde[171] have studied the CD of Cu^{++}-ribonuclease complexes. Copper interaction with ribonuclease is manifested by enzyme inhibition[173] and effects on histidine carboxymethylation.[174] At pH 7 in 0.16 *M* KCl the addition of copper to ribonuclease results in the appearance of a broad negative band that is increased in intensity and changed in shape as successive coppers are added. The CD spectra may be analyzed in terms of two

types of binding sites: a single strong site with a CD minimum at 710 nm and four weaker sites with CD minima near 600 nm. The binding constants obtained agree with equilibrium binding techniques. Carboxymethylation of one histidine results in the loss of one of the weaker sites. In 0.01 *M* salt only the 600-nm band is seen. At pH 9.6 saturation of the CD spectrum does not occur until about 33 sites have been filled and the spectrum shifts to 530 nm, which the authors take as a sign of tetracoordination. Additional structure in the CD is now present in the form of a negative band at 355 nm, and for the first two Cu^{++} ions added, a positive band at 480 nm. At pH values larger than 7, strong positive bands are observed at 251 and 305 nm, which the authors attribute to charge transfer complexes between copper and the peptide backbone. At pH 5.5 there is virtually no CD ellipticity, suggesting that the binding of copper at low pH is unidentate; moreover, there is no evidence of binding to the protein backbone.

Ligand Conformations on Protein Surfaces

Some ligands are optically inactive in solution but become optically active when bound to proteins. This situation may occur when one conformer of an inherently disymmetric chromophore is preferentially bound to the surface of an enzyme. The study of the CD of an enzyme-ligand complex may help decide which conformer is bound on the enzyme surface. For example, Charney and Bernhard[175] examined the ORD of α-chymotrypsin covalently bound with an acyl derivative, β-2-furylacryloyl-α-chymotrypsin. The derivative has a Cotton effect in the region of the furylacryloyl chromophore. From the sign of the Cotton effect the authors suggest that the derivative on the enzyme is in the s-*cis* conformation, while in solution most small model compounds exist in the s-*trans* configuration. Honig et al.[176] suggest that the optical activity of the visual pigment of rhodopsin may be partially explained in terms of a selective binding of a particular conformer by the protein.

In an elegant study, Wicken and Woody[177] have examined the circular dichroism of liver-alcohol dehydrogenase complexes with auramine O. The complex has CD associated with the dye absorption bands. The sterically required nonplanarity of auramine O makes it a disymmetric chromophore, and presumably only one enantiomer is bound to the enzyme. Comparison of the observed CD with theoretical calculations (molecular orbital calculations using a Pariser-Paar-Pople self-consistent field approach as described by Weiss et al.[178]) indicates that the bound enantiomer has its phenyl rings twisted in a left-handed sense through an angle probably not exceeding 45°. Ternary complexes of the enzyme-auramine O, NAD+, NADH, and coenzyme fragments have also been studied.[179] Only minor changes in the dye CD bands are observed on binding coenzyme fragments, but large changes are seen when the complete coenzymes are bound. The authors suggest that the intact coenzymes trigger a conformational change in the liver-alcohol dehydrogenase.

FLUORESCENCE SPECTROSCOPY

Fluorometry is an exceedingly useful tool for the measurement of protein-ligand interactions. First, it is a very sensitive technique and allows accurate studies of proteins in the micromolar concentration range. Second, it is a very versatile tool, and there are several parameters that one can measure which will yield information about the protein. The intrinsic fluorescence of a protein can be characterized by several measurements. The parameters include the wavelengths of maximal activation and emission, the quantum yield and fluorescent lifetime, and the degree of polarization of the fluorescence when the protein is excited with polarized light. In addition, ligands that interact with proteins may themselves be fluorescent, and one may measure the parameters of the ligand. Several molecules also become fluorescent when bound to a protein and may be used as probes of the protein's conformation. There are several excellent reviews in the literature of fluorescence and its application to protein research.[180-189]

Fluorescence is the process whereby light is absorbed at one wavelength and emitted at another. The activation spectrum is the observation of fluorescence at a fixed wavelength when the absorption spectrum is scanned. The emission spectrum is the observation of fluorescence as a function of wavelength when the protein is excited at a fixed wavelength. Fluorescence persists from about 10^{-9} to 10^{-8} sec after excitation. The quantum yield of fluorescence is defined as the ratio of the number of quanta of radiant energy emitted to the number of quanta absorbed.

If a molecule has an even number of electrons, its normal ground state is a singlet, S_0. When visible or UV light is absorbed the molecule is excited to a higher electronic state. The absorption band at longest wavelength usually corresponds to the excitation from the singlet ground state, S_0, to the lowest excited state, S_1. The transition may reach any of the vibrational sublevels of S_1. Within 10^{-13} to 10^{-12} sec some of the excitation energy is dissipated to the surroundings so that the excited molecules reach the lowest vibrational state of S_1. The molecule then returns to the ground state either by a direct transition, accompanied by light emission in the form of fluorescence, or a radiationless transition, in which case the excitation energy is transformed into thermal energy. The mechanism of fluorescence emission is the reverse of excitation, and the molecules may end up in any one of the vibrational states of S_0. Since some energy is dissipated by radiationless transitions, emission occurs at lower energy (higher wavelengths) than the absorption. The emission spectral band often resembles a mirror image of the absorption band of longest wavelength. Occasionally, a molecule may be excited to the triplet state. Triplet-singlet transitions are forbidden by rules of spectroscopy and therefore are very slow, on the order of 10^{-4} to several seconds. The process is called phosphorescence.

There are several processes which cause a loss of fluorescence. Quenching is nonradiative deactivation of the excited state. Mechanisms of quenching include photochemical reactions with the formation of radicals and collisional interactions with other molecules in solution. Another mechanism for the loss of fluorescence at a particular wavelength is excitation transfer. When there is a good overlap between the emission bands of the primary absorbing molecule (called the sensitizer) and the absorption band of another molecule (called the acceptor), excitation transfer may occur. If the second molecule is nonfluorescent, it will act solely as a quencher. If the acceptor is itself fluorescent, the transferred energy may be emitted with the spectrum characteristic of the acceptor.[121] A theory developed by Förster[190] predicts that the efficiency of transfer will be proportional to both the overlap integral between the emission and absorption bands and the reciprocal of the sixth power of the linear distance between the donor and acceptor. Thus, energy transfer may be used to measure distances between donor and acceptor molecules. Energy transfer processes in enzyme-ligand complexes will be discussed in greater detail below.

If the exciting light is polarized with its electric vector in the vertical direction, molecules oriented with their transitions in this direction will be preferentially excited. When the molecules move slowly they will emit light before they change their orientation. The light will, therefore, be partially polarized when viewed in a horizontal direction. This phenomenon is called polarization of fluorescence. The degree of polarization is

$$(p) = \frac{I_v - I_h}{I_v + I_h} \tag{36}$$

where

I_v = intensity of the components with electric vectors oriented in a vertical direction;
I_h = intensity of the components with electric vectors oriented in a horizontal direction.

Polarization of fluorescence is also a useful technique for measuring enzyme-ligand interactions.

Enzyme-Ligand Interactions

Effects on the Intrinsic Fluorescence of Proteins

Chen[186] has defined protein fluorescence with terms analogous to those describing Cotton effects observed in the study of the ORD and CD of proteins. Aromatic amino acids present in proteins endow them with an intrinsic UV fluorescence. Fluorescence due to bound chromophores (either covalently or noncovalently bound) is called extrinsic flourescence.

The intrinsic fluorescence of proteins is mainly due to tyrosine and tryptophan residues; however, the fluorescence of proteins is not quantitatively related to the tryptophan and tyrosine content. Usually the quantum yields for tyrosyl residues are far lower than the yield for the free amino acid. For a while it was disputed whether proteins containing both tyrosine and tryptophan showed tyrosine fluorescence at all.[191-195] Konev[191] found fluorescence due to tyrosine in some tryptophan-containing proteins while Velick[192] and Weber[193,194] did not detect it. Subsequently, Weber[195] showed that one could separate the tyrosine contribution to the fluorescence by exciting at two different wavelengths. When proteins are excited at 292 only trytophan fluorescence is noted. If proteins are excited at 278, however, the

tyrosine fluorescence appears as a shoulder on the tryptophan band. Udenfriend[188] has reviewed the mechanisms which may cause low quantum yield for tyrosine residue. These mechanisms include quenching by ions such as hydroxyl, hydrogen, and phosphate ions, quenching by the peptide bond and carboxyl groups, and transfer to tryptophan residues in the protein. Tryptophan residues, on the other hand, have quantum yields either higher, lower, or about the same as the free amino acids, and the emission maximum varies depending on which protein is studied.[180,191,193,194]

The intrinsic fluorescence of proteins may be quenched or enhanced when the proteins interact with ligands. The changes may be used to follow the extent of ligand binding. Some of the early studies utilizing the quenching of protein fluorescence to measure enzyme-ligand complexes have been reviewed by Shifran and Kaplan[51] and Udenfriend.[183,188] For example, in a classic paper Velick[192] showed that the binding of NADH to both lactic dehydrogenase and triosephosphate dehydrogenase causes quenching of their intrinsic fluorescence. The loss of fluorescence was used by Velick to estimate the binding constants for the coenzymes to the enzyme.

Recently there have been several detailed studies on the fluorescence of proteins which have yielded information about enzyme-ligand interactions. For example, the lysozymes have been extensively studied, and the fluorescence properties of several lysozymes have been reviewed by Imoto et al.[120] and Koenig and Longworth.[196]

Hen egg white lysozyme has six tryptophans, three of which are thought to be in the active site.[197] In an early study Shinitzky et al.[198] examined the change in fluorescence of a number of inhibitory sugars and found that strong inhibitors caused a shift in the emission maximum to shorter wavelengths and an increase in the height of the emission peak. The blue shift implies a change of the tryptophan environment to a more hydrophobic one. Lehrer and Fasman[199-201] studied the fluorescence of lysozyme upon the binding of *N*-acetyl-glucosamine and the di- and tri-oligomers of the sugar and found a progressive enhancement and shift to lower wavelength with the increasing size of the inhibitors. They concluded that some groups become less available for quenching the indole rings as the inhibitors bind. Furthermore, they showed a dependence of the enhancement of fluorescence upon the pH of the solution. While the enzyme fluorescence alone shows little change up to pH 8, the trioligosacharride complex shows a marked increase in fluorescence between pH 2.5 and 4.5 and between pH 5.5 and 7.5 with apparent pK's of 3.5 and 6.5. Since unionized carboxyl groups are known to quench fluorescence, the results are consistent with two carboxyls, one with an abnormally low pK and the other with an abnormally high pK being responsible for the quenching of tryptophan fluorescence. Lehrer and Fasman also used fluorescence to calculate the binding constant of tri-*n*-acetyl-glucosamine to lysozyme as a function of pH and implicated two groups with pK's of 6.2 and 4.2 in the binding.[200]

Bablouzian et al.[202] described a differential spectrofluorometer which they have used to try to separate the contributions of different tryptophans from the spectrum of hen egg white lysozyme (see Figure 9). More recently, specific chemical modifications have been used to assign the protein fluorescence to specific residues. These studies have been summarized by Imoto et al.[120] The total fluorescence of a protein in the absence of energy transfer is expected to be the sum of the contributions of the individual tryptophan and tyrosine chromophores; however, it appears that tryptophans 62 and 108 represent essentially all the fluorescence of the free protein. Modification of lysozyme to the dioxindole derivative where tryptophan 62 and 108 are oxidized results in destruction of all but 10 to 15% of the native fluorescence. Oxidation of all six tryptophans reduces the relative fluorescence to 5% of the native fluorescence. The two tryptophans, moreover, do not contribute in a simple additive way to the total fluorescence, as shown by preparation of derivatives with only one tryptophan oxidized.

Teichberg and Sharon[203] compared the fluorescence of native lysozyme with lysozyme which has tryptophan 108 oxidized to the ester with glutamate 35. From a study of the modified protein they deduced that the binding of tri-*n*-acetyl glucosame results in a conformational change in which glutamate 35 is separated from tryptophan 108. Imoto et al.[120] note that no such movement is observed in the X-ray analysis, and if such a change does occur, it is very small (less than 0.5 Å).

While the interpretation of the exact cause of fluorescence changes is difficult, the technique still can give some unambiguous answers. For example,

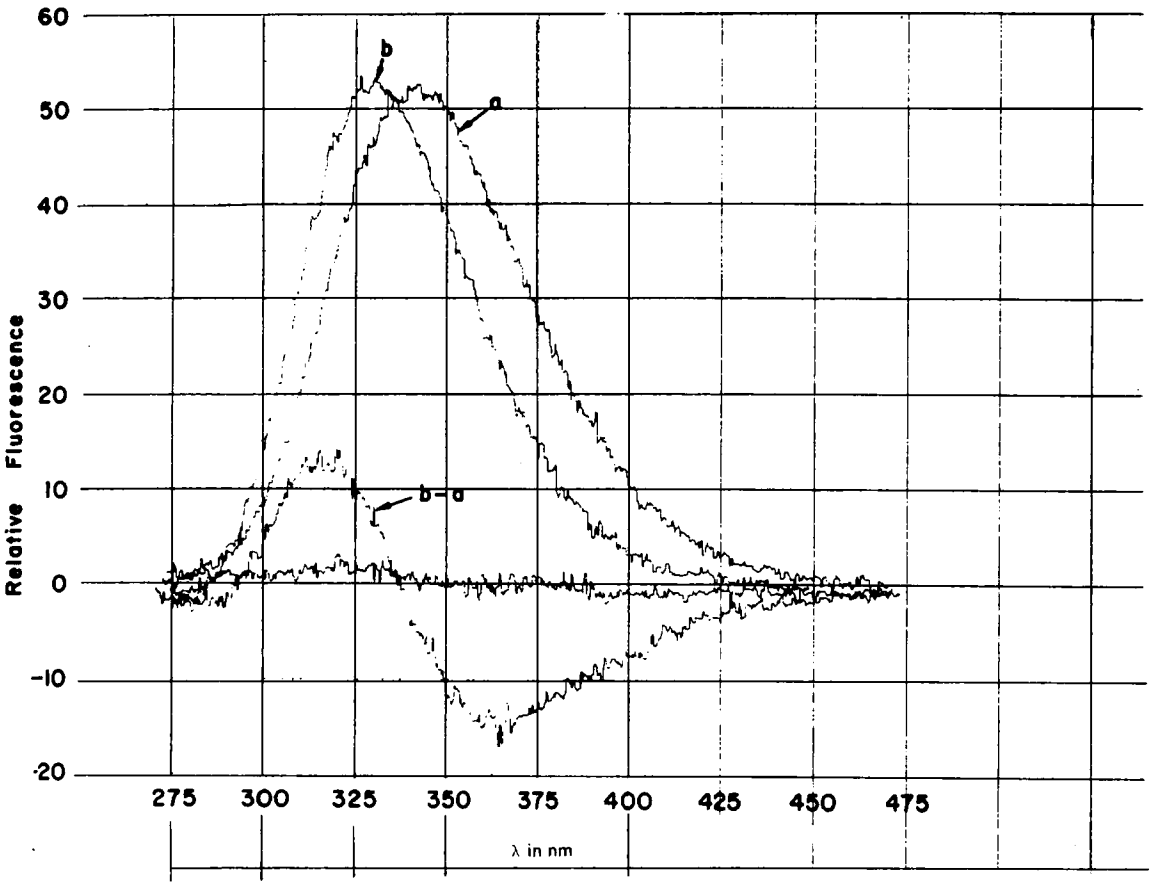


FIGURE 9. The fluorescence spectra of lysozyme and inhibitor in water at pH 5.1. (a) Pure enzyme; (b) With tri-*n*-acetyl-glucosamine; (b-a) The difference spectra. (From Bablouzian, B., Grourke, M., and Fasman, G. D., *J. Biol. Chem.*, 245, 2081, 1970. With permission.)

Secemski et al.[204] have recently used the intrinsic fluorescence of lysozyme to examine the binding of a σ-lactone derived from tetra-*N*-acetylchitotetraose to lysozyme. The compound appears to be a "transition state" analog of the substrate. The association constants for the binding of the analog to lysozyme were obtained from fluorescence measurements. The pH dependence of the binding shows that the strongest analog binding occurs with the species of lysozyme in which the carboxyl group of glutamate 35 is dissociated. In the enzyme analog complex the pK of glutamate 35 is 4.7, while it is 6.0 in the enzyme alone.

Mulvey et al.[61] have studied the spectral properties of human lysozyme. The spectrofluorometric titration of human lysozyme is very similar to hen egg white lysozyme. In the presence of oligomers of *N*-acetyl-D-glucosamine, however, there is no quenching of fluorescence below pH 5.5 as found in the hen egg white lysozyme due to the substitution of a tyrosine in the human enzyme for the corresponding tryptophan in the binding site of the hen egg white enzyme.

Extrinsic Fluorescence of Proteins

When small molecules bind to proteins there may be changes in the quantum yield or fluorescence polarization. The small molecules may be normally fluorescent or may become fluorescent when bound to the protein. An example of the former type is NADPH. An example of the latter type is a class of dyes which are nonfluorescent in aqueous solution but become fluorescent in less polar solvents. These dyes are used as probes of hydrophobic pockets of proteins and include 1-anilo-8-naphthalene sulfonate,[205] 2-*p*-toluidnyl-naphtalene-6-sulfonate,[206] and tetracycline.[207]

Binding of Fluorescent Ligands to Proteins

Fluorometry has been widely used to study the interactions of fluorescent coenzymes with enzymes. NADH and NADPH, for example, are naturally fluorescent. In 1956 Boyer and Theorell[208] showed that the coenzyme complex of alcohol dehydrogenase showed an intensification and shift in the fluorescence spectrum of the reduced pyridine nucleotide cofactor. This property is often a general phenomenon of dehydrogenases.[183] Fluorometric studies of enzyme-coenzyme complexes have been reviewed.[51,183] Besides the reduced pyridine nucleotide cofactors, several other coenzymes are naturally fluorescent and may be used to measure enzyme-coenzyme interactions. These include pyridoxal phosphate, flavins, chlorophyll,[183] and porphyins.[209] When fluorescent ligands bind to proteins their fluorescence may be quenched or enhanced. Moreover, where the small ligands bind to the relatively large proteins there may be changes in the polarization of the fluorescence, which may be used to measure the interactions. As an illustration, Dugan and Porter[210] have utilized fluorescence enhancement, fluorescence polarization, and equilibrium dialysis to monitor the binding of NADH to mammalian and avian fatty acid synthetases. Their study is one of many good illustrations of the use of ligand fluorescence. All three methods show that the enzyme from pigeon liver has two coenzyme sites in 0.2 *M* phosphate buffer, but three binding sites were determined from the fluorescence measurements in 0.01 to 0.1 *M* phosphate or histidine buffer. On the other hand, the fluorescence and fluorescence polarization methods showed four binding sites for the enzyme from rat liver in phosphate buffer from 0.025 to 0.2 *M*. The authors also used the fluorescence techniques to determine the dissociation constants of the enzyme-coenzyme complexes. The pigeon liver enzyme had a single constant for all the sites which was dependent upon ionic strength, while the rat liver enzyme had two classes of binding sites whose strengths were also a function of ionic strength.

Energy Transfer

As described above, when light is absorbed by one chromophore but emitted by another, energy transfer is said to occur. The transfer of singlet-singlet electronic excitation energy can be transferred over distances on the order of 30 Å. Förster[190] has proposed that the transfer occurs by a dipole-dipole resonance interaction between the energy donor and acceptor chromophores. In his treatment the rate constant for transfer is proportional to the inverse sixth power of the distance between the groups. It is also proportional to the mutual orientation of the groups, the refractive index of the solvent, and the overlap of the emission spectrum of the energy donor and the absorption spectrum of the energy acceptor. If the latter factors can be determined, energy transfer, in theory, allows measurements of the distance between two fluorescent groups and may be used as a "spectroscopic ruler."[211] Several groups[211,212] showed that Förster's theory is valid in model systems. The topic of long-range energy transfer in proteins has been reviewed by Steinberg.[213]

Eisinger et al.[214] have critically discussed the application of Förster's theory to the study of proteins and polypeptides. The rate of Förster-type energy transfer from an excited donor, D, to an acceptor, A, is given by:

$$K_{AD} = \frac{8.8 \times 10^{-25} \phi_o K^2 J_{AD}'}{n^4 \tau_D r^6} \quad (37)$$

with

$$J_{AD} = \int_o^\infty F_D(v)\, E_A(v)\, v^{-4}\, dr \quad (38)$$

where

- τ_D = donor emission lifetime;
- ϕ_o = quantum yield;
- n = index of refraction of the medium intervening between the donor and acceptor at a wavelength in the region of their spectral overlap;
- K^2 = dipole-dipole orientation factor;
- r = donor-acceptor separation;
- $\epsilon_A(v)$ = decadic molar extinction coefficient of the acceptor
- $F_D(v)$ = spectral distribution of the donor emission normalized to unity;
- J_{AD}' = overlap integral between ϵ_A (v) and F_D (v) modified by the frequency factor, v^{-4}.

The distance, R_o, at which the rate of energy transfer is equal to the sum of all other modes of deexcitation of the donor is called the Förster critical distance and is given by

$$R_0^6 = 8.8 \times 10^{-25} \phi_D k^2 n^{-4} J_{AD}' \quad (39)$$

If the distance between a donor and acceptor is r, the efficiency of transfer between them is given by

$$e = \frac{r^{-6}}{r^{-6} + R^{-6}} \quad (40)$$

Thus, one may estimate distances by both the rate and efficiency of fluorescence transfer.

In order to evaluate R_0 and, therefore, distances, one must determine J_{AD}' and be able to evaluate ϕ_D, k^2, and n^4. Eisinger et al.[214] have calculated the values of J_{AD} for many donor-acceptor pairs found in proteins. In addition, they have suggested reasonable ways of estimating ϕ_D, k^2, and n^4.

A. The quantum yield of the donor, ϕ_D, may sometimes be measured directly or an average value for the typical chromophore may be used.

B. The orientation factor is k^2. If the donor and acceptor transitions have a fixed but unknown orientation with respect to each other, θ_{AD}, and make angles ϕ_D and ϕ_A with the line joining them, k is given by $k = \text{Cos}_{AD} - 3 \text{ Cos } \theta_A \text{ Cos } \theta_D$. Therefore, k^2 may vary between 0 and 4. Since the angles are generally not known, Eisinger et al.[214] suggest that .475 be used as an average value for k^2 with random orientation of the donor and acceptor molecule in a rigid solution. Stryer and Haugland[211] suggest that the value of k^2 = 2/3 should be used if the groups are rotating freely. In most proteins, however, the fluorescence groups will have a fixed nonrandom orientation of the groups and, thus, there may be a large error in the estimation of R_0 and r.

C. Eisinger et al.[214] suggest that the index of refraction may be approximated by the index of refraction of water in the wavelength region of spectral overlap of donor and acceptor. At 300 nm, n = 1.5. Another source of error in measurements of distances from energy transfer is the possibility that the distance between the donor and acceptor will change during the donor's fluorescent lifetime. Eisinger et al.,[214] however, calculate that this error is small.

Various authors have estimated J_{AD} for various pairs of donors and acceptors commonly found in proteins; their findings have been summarized by Steinberg.[213]

The Use of Energy Transfer in Measurements of Enzyme-Ligand Interactions

Because of the uncertainty in the orientation factor, precise estimates of distances between fluorescent ligands on a protein may be difficult to obtain; however, some very useful information may be obtained from a study of energy transfer in protein-ligand complexes. Many interesting studies have been summarized by Steinberg.[213] For example, the activation spectrum of the NADH fluorescence in glyceraldehyde-3-phosphate dehydrogenase exhibits a peak at 290 nm which is absent in free NADH. Thus, energy transfer from tryptophan to NADH has been used by Velick[192] to explain the partial quenching of the enzyme by the cofactor. Transfer of electronic excitation energy from the tryptophan residues to the bound NADH is also found with lactic dehydrogenase.[191] Since there are a large number of tryptophans in the enzymes and the R_0 value for tryptophan-NADH energy transfer is large, but only a small amount of energy transfer is observed, it is likely that the tryptophan residues are distributed and oriented in fashions unfavorable for energy transfer to the coenzyme.

Steinberg[213] has also reviewed studies of energy transfer in heme proteins,[215,216] phycocyanin,[217] pyridoxyamine-5-phosphate and pyridoxyl-5-phosphate-containing enzymes,[218-221] transferrin,[222,223] and hemocyanin.[224,225] Some other recent examples illustrating the uses of energy transfer in enzyme-ligand interactions are detailed below. For example, Luk[226] has estimated the distance between the metal-binding sites in transferrin using trivalent lanthanide ions as fluorescent probes. Human serum transferrin is an ion-binding protein which acts as an ion buffer and carrier. It has two iron-binding sites which can also bind other metal ions. It was found that there are two specific binding sites per transferrin molecule for Tb^{3+} Eu^{3+} Er^{3+} and Ho^{3+} but only one site for Nd^{3+} and Pr^{3+} which have large ionic radii. The fluorescence and difference spectral studies suggest that the terbium ion is bound to the phenolic oxygen of the tyrosyl residues. First, the difference spectrum shows the characteristic spectrum of a tyrosinate ion. Second, fluorescence of tyrosine in the molecule is very efficiently quenched by Tb^{+3} and the Tb^{+3} fluorescence is enhanced greatly, suggesting very efficient energy transfer. From the lack of energy transfer between Tb^{+3} and Fe^{+3}

ions bound to the same protein, the author suggests that the distance between the binding sites is equal or greater than 43 Å.

Lehrer and Kerwar[227] have studied the effects of Cu^{++} on intrinsic fluorescent of G and F actin. When equimolar Cu^{+2} is bound to G or F actin about 75% of the tryptophan fluorescence is quenched. From the magnitude of the parameters the mechanisms appear to involve long-range energy transfer. From the magnitude of quenching it can be calculated that more than one tryptophan residue is quenched. Since there is no significant difference in the tryptophan fluorescence between Cu-G and Cu-F actin and no loss of fluorescence occurs upon polymerization of Cu-G actin, the authors conclude that the copper-binding site is probably located at least 20 Å (the critical transfer distance) from tryptophan side chains of neighboring monomers of F actin.

An interesting study utilizing energy transfer is a paper on the binding sites of the pyruvate dehydrogenase multienzyme complex.[228] Thiochrome diphosphate and flavin adenine dinucleotide bind to the active sites of the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase enzymes of the pyruvate dehydrogenase multienzyme complex. Results from both steady-state and nanosecond lifetime fluorescence measurements are consistent with a decrease in both the quantum yield and fluorescence lifetime of enzyme-bound thiochrome diphosphate when the flavin is present at its binding sites. When the results are analyzed in terms of an energy transfer mechanism, the measured efficiency of transfer is found to be 7.8%. This corresponds to an apparent distance between sites of approximately 45 Å. Uncertainty concerning the orientation of the dipoles of the donor and acceptor suggest that the possible range of distance is 30 to 60 Å. The absorption and fluorescence properties of the flavin are not altered by the binding of ligands at the active sites of the other enzymes in the complex, indicating that there is very little interaction between the active sites of the different enzymes in the complex.

Singlet-singlet energy transfer has been most extensively studied; however, triplet-triplet[229] and triplet-singlet[230] energy transfer may also be of some used as a criterion of proximity in proteins. While singlet-singlet energy transfer may be used to measure distances in the 10 to 60 Å range, triplet-triplet transfer can be employed to measure distances in groups which are closer together, on the order of 12Å. For example, Galley and Stryer[229] studied the binding of *m*-acetylbenzenesulfonamide to carbonic anhydrase. The sulfonamide is the triplet donor and tryptophan residues in the protein are triplet acceptors. The sulfonamide is an inhibitor which binds to the zinc atom of carbonic anhydrase. Very efficient energy transfer was observed, suggesting that there is a tryptophan residue close or in the active site on the protein. In contrast, energy transfer was not observed in a derivative of α-chymotrypsin, suggesting that tryptophan is absent from the portion of the active site probed by the donor.

Galley and Stryer[230] demonstrated triplet-singlet energy transfer between a complex of proflavin and α-chymotrypsin. Triplet-singlet energy transfer, like singlet-singlet, occurs over the order of 40 Å. The tryptophan residues were the triplet donors while the proflavin bound at the active site served as the singlet acceptor. The occurrence of triplet-singlet energy transfer was shown by the delayed fluorescence of the bound proflavin and selective quenching of the tryptophan phosphorescence. The kinetics of the energy transfer revealed that there are at least two classes of tryptophan residues in the molecule.

Fluorescent Probes

The application of fluorescence spectroscopy to protein research has been greatly extended by the use of fluorescent probes. Among the commonly used probes are fluorescent analogs of naturally occurring substrates and cofactors of enzymes, dyes which become fluorescent when bound to the surface of proteins and may be used as indicators of hydrophobic areas of protein surfaces, and fluorescent covalent labels attached to specific sites on a protein. The topic of fluorescent probes has been reviewed recently.[231]

Fluorescent analogs – It is possible to make a fluorescent analog from a naturally occurring substrate in several ways. The first is to chemically modify the naturally occurring substrate. An example of this is the synthesis of fluorescence analogs of adenine containing coenzymes by the reaction of the coenzymes with chloroacetaldehyde.[232-234] The fluorescence analogs are often enzymatically active and, thus, serve as useful probes of the cofactor binding site. The second method commonly used is to attach a fluorescent

molecule covalently to the compound of interest. For example, Whiteley[235] has prepared a fluorescein analog of folic acid.

Noncovalently bound fluorescent probes – Fluorescent probes are small molecules which undergo changes in their fluorescent properties as a result of noncovalent interaction with a protein or other macromolecules. Among the most popular probes used are 1-anilino-8-naphthalene sulfonate (ANS) and related compounds. Weber and Laurence[236] first showed that these dyes had only weak green fluorescence in water, but gave intense blue fluorescence when bound to proteins. Subsequently, these dyes were used extensively as probes of protein structure. The use of these probes has been reviewed.[189,206,231,236-240] Recently, Brand and Gohlke[231] summarized many studies illustrating the use of these probes; thus, only selected examples will be given here.

One of the first studies showing that nonspecific dyes could be used as probes of specific binding sites was Stryer's work on the interaction of 1-anilino-8-naphthalene sulfonate with apohemoglobin and apomyoglobin.[205] One mole of ANS is bound per mole of apoprotein with a dissociation constant of 10^{-5} *M*. The proteins which bind heme do not bind the dye, and heme displaces the dye from its complexes, suggesting that the dye and heme are bound in the same sites. When the dye is bound the quantum yield increases 200-fold and the emission peak is shifted from green to blue by 60 nm. The changes were attributed by Stryer[205] to the nonpolar environment of the heme binding site, as organic solvents had similar effects on the fluorescence of the naphthalene dye.

Appropriate dyes may serve as "specific" fluorescent probes of binding sites. For example, Chen and Kernohan[241] describe the combination of bovine carbonic anhydrase with a fluorescent sulfonamide. 5-Dimethylaminonaphthalene-1-sulfonamide binds to carbonic anhydrase. Quenching of the protein fluorescence and enhancement of the dye emission may be used to calculate a dissociation constant of 2.5×10^{-7} *M*. The fluorescence of the free dye has a peak emission at 580 nm and a quantum yield of 0.055, but the bound dye has an emission maximum at 468 nm and a yield of 0.84. The authors suggest once again that this shift is due to the hydrophobic environment of the binding site. The binding appears to specifically involve the sulfonamide site known to exist in carbonic anhydrase, since other fluorescent probe compounds show no evidence of binding to the enzyme. Calculations of energy transfer efficiency show that 85% of the tryptophan absorption is transferred to the dye. The authors suggest that the dye is within the critical transfer distance of 21 Å from all the trytophan molecules. The tryptophan fluorescence, however, is only 73% quenched by the binding of 1 mol of dye. This degree of quenching is less than the overall efficiency of transfer, suggesting that the fluorescence efficiencies of the 7-tryptophans in the protein are different and that the dye is bound in such a way that energy transfer occurs with greater probability from those residues which are relatively less fluorescent. The authors also studied the decay time of the dye fluorescence and concluded that the relaxation time is consistent with a low degree of molecular asymmetry.

Another recent study[242] illustrates the use of "specific" active site-direct fluorescent probes of horse serum cholinesterase. 1-(5-Dimethylaminonaphthalene-1-sulfonamideo)3-*N*,*N*-dimethylaminopropane (an aminosulfonamide) and *N*-methylacridinium iodine (a heterocyclic aromatic quaternary salt) were used as probes of the anionic subsite of purified horse serum cholinesterase. *N*-Hydroxynaphthalimide-diethyl-phosphate (the insecticide Maretin®) was used as a fluorescent probe of the esteritic subsite. Diisopropyl phosphorofluoridate and Maretin® give identical numbers of esteritic sites in the enzyme by titration. The aminosulfonamide and Maretin® compete for a common hydrophobic area distinct from the hydrophobic area which binds the *N*-methylacridinium ion. The aminosulfonamide and *N*-methylacridinium ion, however, compete for the anionic subsite in the enzyme. The Förster distances for 50% energy transfer from tryptophan residues measured with the bound *N*-methylacridinium ion and the aminosulfonamide are 19 and 17 Å, respectively.

Fluorescent probes bound at some distance from the active site of a molecule may also be useful indicators of enzyme-ligand interactions. For example, McLaughlin[243] has recently studied the interaction of 8-anilino-1-naphthalene sulfonate with creatine kinase. Creatine kinase has one tight binding site for ANS per subunit with an enhancement of fluorescence of 100-fold and a blue shift of 30 nm. Nucleotide substrates

noncompetively interact with the dye-binding site, causing a 50% decrease in the fluorescence of the bound dye and a shift of the emission maximum of 7 nm, while quanido substrates alone do not affect the fluorescence of the bound dye. The fluorescence of the bound dye was used by McLaughlin[243] to determine the dissociation constants of nucleotide substrates from the enzyme in the presence or absence of other substrates or effectors. The results gave evidence for cooperativety of nucleotide binding.

Covalently bound fluorescent labels – Fluorescent probes are nonconvalently bound to proteins. It is possible, however, to prepare compounds that can be covalently attached to specific residues on the molecules. These labels can be "active site directed" or be aimed at the modification of a certain type of residue. For example, Kenner et al.[244-246] developed a procedure for the specific conversion of tyrosine to aminotyrosine which then can be specifically dansylated to give a fluorescent derivative. Fluorescent labels have also been prepared that are specific for cysteine thiols[247,248] and amino groups.[249]

Once the proteins have been labeled, the fluorescent probe can serve as a reporter group. For example, Steiner et al.[250] prepared the dansylamino tyrosine derivative of soybean trypsin inhibitor. The protein has two tyrosine groups which were derivatized. The fluorescence properties of the two dansylaminotyrosine groups responded differently to reaction with trypsin and KI quenching.

Recent examples of the use of fluorescent labels are in a study by Wu et al.[251] on the conformational transitions of cyclic adenosine monophosphate receptor protein of *E. coli*. In their study the protein was labeled covalently with two fluorescent reagents, *N*-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate and dansyl chloride. Both the naphthylamine derivative and the dansylated protein are fully active in binding cyclic adenosine monophosphate (cAMP). When cAMP is added to a solution of the protein labeled with the naphthylamine, there is an increase in fluorescence intensity and a blue shift of the emission maximum of the label, indicating a conformational transition of the protein. Biologically active analogs of cAMP also induce this change, but cyclic nucleotides such as cGMP and 1,N^6-etheno-cAMP, which are competitive inhibitors of cAMP, do not. The authors related the changes observed to the cAMP-dependent gene transcription. The authors also found another specific conformational change of the receptor protein when the protein interacts with the lac operon in the presence of cAMP. This is demonstrated by a cAMP-sensitive blue shift and a quenching of the emission spectrum of the naphthylamine derivatized protein upon binding of λh80d lac DNA, but not to λh80 DNA. Nanosecond fluorescence depolarization studies of the dansylated receptor protein showed that cAMP addition does not cause association or dissociation of the receptor protein.

CONCLUSION

As shown above, the various forms of spectroscopy can be exceedingly useful tools for examining protein-ligand interactions. It is evident that the forms complement one another, and for maximum information about protein-ligand interactions the techniques should be used in concert.

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