Circular Dichroism and Fluorescence Studies on the Binding of Ligands to the α Subunit of Tryptophan Synthase†

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ABSTRACT: Binding to the α subunit of tryptophan synthase induces extrinsic Cotton effects in the substrates indole (IND), indoleglycerol phosphate (IGP), and D-glyceraldehyde-3-P (D-GAP) and in the inhibitor indolepropanol phosphate (IPP). These effects disappear when the enzyme is denatured in guanidinium chloride. The induced circular dichroism (CD) was used to determine the dissociation constant and the number of binding sites for IPP. The dissociation constant so determined is equal to 48 μM and is in good agreement with the value of 48 μM obtained by equilibrium dialysis. From the temperature dependence of the dissociation constant, a value of -2.8 kcal/mol for the binding enthalpy was obtained. The determination of dissociation constants by means of extrinsic Cotton effects is shown to be quite feasible. CD competition experiments with glycerol phosphate (GP) suggest that IPP binds bifunctionally to the enzyme: via its indole part and its phosphate group. Indolepropanol, which lacks the phosphate group, does not show an extrinsic Cotton effect. Since the induced CD is strongly dependent on the binding geometry, the close similarity between the induced spectra in IPP and IGP is additional evidence that IPP is a good substrate analog. Binding to the enzyme results in a blue shift of the IPP fluorescence emission maximum. The dissociation constant determined by fluorescence titration equals 46 μM and agrees well with the values determined by the other two methods. Previous biochemical and fast kinetic studies suggested the existence of multiple conformational states for the enzyme and of ligand-induced conformational changes. No evidence was found in the far-uv CD spectra for conformational changes upon binding of IND and D-GAP. For IPP a very small effect was observed.

The α subunit of tryptophan synthase (L-serine hydrolyase (adding indole) (EC 4.2.1.20)) from *Escherichia coli* (mol wt 29,000) catalyzes the reaction

$$\underset{\text{(IGP)}}{\text{indoleglycerol phosphate}} \rightleftharpoons \underset{\text{(IND)}}{\text{indole}} + \underset{\text{(D-GAP)}}{\text{D-glyceraldehyde-3-P}}$$

The literature on this enzyme was recently reviewed (Yanofsky and Crawford, 1972). Indolepropanol phosphate (IPP)[1] cannot undergo aldolytic cleavage at the 3–3′ bond and is known to be a strictly competitive inhibitor with respect to IGP (Kirschner and Wiskocil, 1972).

A number of optically inactive molecules become optically active when bound to proteins (Chignell, 1972; Blauer, 1974). These induced Cotton effects are due to the interaction of the chromophore with the asymmetric environment provided by the protein. The indole transitions of IGP, IND, and IPP are expected to be optically inactive for the free ligands. If extrinsic Cotton effects in the $\pi \rightarrow \pi^*$ transitions of the indole chromophores occur upon binding, they are expected to appear in the wavelength range where each of these chromophores absorb. Fortunately, the α subunit of tryptophan synthase contains no tryptophan, which normally makes the largest contribution to the near-uv protein CD and which absorbs in the same wavelength region as the indole chromophore. This protein thus offers the possibility of detecting CD effects in the near-uv induced in the indole chromophores of various substrates and inhibitors. The weak CD background due to the tyrosine and phenylalanine residues of the protein is expected to occur mainly at lower wavelengths. The enzyme contains no disulfide bridges which would otherwise also contribute. If the induced effect is sufficiently large, it can be used for the determination of binding constants. Although binding constants have been determined in this way before (Devaux et al., 1974; Greenfield et al., 1972), few quantitative analyses exist, in which the results are also compared with those from other methods.

Little is known about the mode of binding of these ligands to the enzyme. Such information is of value in the elucidation of the reaction mechanism and in the interpretation of fast kinetic experiments. Extrinsic Cotton effects, because of their great sensitivity to binding geometry, may be of use in this respect. It may be expected, for instance, that if the competitive inhibitor IPP is a good substrate analog and binds in the same way as the substrate IGP, the induced extrinsic effects will be very similar for both ligands. The substrate IGP and the inhibitor IPP have at least two functional groups for binding to the protein: the hydrophobic indole part and the charged phosphate group. These two points of attachment would allow for a rigid anchoring of the ligand to the protein. Such a complex seems to be required to explain a large induced effect (Chignell, 1972). Assuming this bifunctional binding model to be correct for a moment, we expect strong binding of IPP and IGP and significant induced CD effects, whereas the ligands IND and indolepropanol (IPL), which lack the phosphate group, should show much weaker binding and much smaller CD effects. Further evidence for binding via the phosphate group was sought from CD competition experiments between IPP and phosphate-containing ligands.

Since the protein contains no tryptophan, the change in

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[1] Abbreviations used are: IND, indole; IGP, indoleglycerol phosphate; IPP, indolepropanol phosphate; IPL, indolepropanol; D-GAP, D-glyceraldehyde 3-phosphate; GP, glycerophosphoric acid or glycerol phosphate; PGA, 3-phosphoglycerate.

indole fluorescence upon binding can be studied in the absence of the normal protein background. It can be used to study the binding via the indole group, and provides an additional method of determining binding constants.

Conformational changes in the α subunit upon binding by IND and IGP have been postulated on the basis of biochemical and kinetic evidence (Freedberg and Hardman, 1971; Kirschner and Wiskocil, 1972; Yanofsky and Crawford, 1972). The possible existence of various conformations of the isolated α subunit also plays an important role in the assembly process of α and β subunits into the multienzyme complex. We studied this question directly by CD measurements in the far-uv region.

Materials and Methods

Buffer. Unless stated otherwise, all experiments were performed in 5×10^{-2} *M* Tris-HCl buffer (pH 7.5) with 10^{-3} *M* EDTA and 2×10^{-4} *M* 1,4-dithioerythritol.

Enzyme. The α subunit of tryptophan synthase was isolated from the *Escherichia coli* trpB8 strain. The purification followed a new method (K. Kirschner et al., to be published) which omits the low pH step of the standard procedure (Henning et al., 1962). The enzyme is stored as a crystalline suspension. Prior to experiments, aliquots were dialyzed against an excess of Tris buffer. The chemical purity of the enzyme was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969). The enzymatic activity was tested by the standard method (Smith and Yanofsky, 1963). The enzyme used for quantitative measurements was chemically pure and had a specific activity between 4700 and 5000 U/mg indicating that it was almost completely active. Protein concentrations were determined spectrophotometrically in 0.1 *M* phosphate buffer (pH 7.6) with 10^{-3} *M* EDTA and 2×10^{-4} *M* 1,4-dithioerythritol, using $\epsilon_{278\ nm} = 1.33 \times 10^4\ M^{-1}\ cm^{-1}$.

Ligands. Indolepropanol phosphate (IPP), indoleglycerol phosphate (IGP), both in the form of their dicyclohexylammonium salts, and indolepropanol (IPL) were kindly supplied by Dr. K. Kirschner. For spectrophotometric determination of concentrations $\epsilon_{H_2O} = 5.4 \times 10^3\ M^{-1}\ cm^{-1}$ was used at 281 nm for IPP, at 278 nm for IGP, and at 280 nm for IPL. The purity of IPP was established by thin-layer chromatography on silica gel, with methanol–ammonia–water (7:2:1) as the moving phase. Indole (IND), analytical grade, was purchased from Merck and used without further purification. The molar extinction coefficient in water was determined to be $5.54 \times 10^3\ M^{-1}\ cm^{-1}$ at 278 nm. D,L-Glyceraldehyde 3-phosphate (GAP) and D-glyceraldehyde 3-phosphate (D-GAP) were prepared from their diethyl acetal Ba salts following the method of Racker (Racker et al., 1959). The complete oxidation with NAD^+ by glyceraldehyde-3-phosphate dehydrogenase was used to determine the concentration of the D isomers in such preparations (Krebs, 1955). D,L-α-Glycerol phosphate (GP), D,L-3-phosphoglycerate tricyclohexylammonium salt (PGA), and guanidinium chloride were of analytical grade and used without further purification.

CD Measurements. Measurements of circular dichroism were carried out with a Cary 61. Thermostatable cells of thicknesses ranging from 25 μm to 5 cm were used. The optical density was kept below 1.5 for all wavelengths. Titration experiments were carried out using a fresh solution for each ligand concentration. Titration data were collected at fixed wavelength, and recorded over extended periods of time. Repeated measurements of buffer signals were taken in order to eliminate the effect of base-line drifts.

Fluorescence measurements were carried out with an RRS 1000 direct recording corrected spectrofluorometer (Schoeffel Instrument Corp., Westwood, N.J.). Spectral bandwidths were 1.6 nm for the excitation beam and 6.4 nm for the emitted radiation; 2×10 mm cuvets, thermostated at 25°, were used in order to keep absorption low even at high ligand concentrations. Fluorescence intensity measurements needed to be corrected for absorption of incident light only at the highest ligand concentrations used. During titration experiments, aliquots of IPP solution were added successively to an initial 600 μl of protein solution, using an Agla micrometer syringe (Wellcome Research Laboratories, Beckenham, England). The IPP aliquots contained small amounts of enzyme in order to keep the enzyme concentration constant during titration. In the reference cuvet the same amount of IPP alone was added to an initial 600 μl of buffer. At each IPP concentration the two signals were recorded at fixed wavelength for several minutes.

Absorption measurements were carried out with a Zeiss PMQ II spectrophotometer or a Cary 15.

Data Analysis. Since the free ligands IPP and IND show no CD, the induced effect is entirely due to the binding, and no corrections have to be made for varying contributions from free ligands. We define R by

$$R = \frac{\Delta\epsilon(S_T) - \Delta\epsilon(0)}{\Delta\epsilon(\infty) - \Delta\epsilon(0)} \tag{1}$$

$\Delta\epsilon\ (S_T)$ is the induced CD effect at fixed wavelength and at the total ligand concentration S_T. It is easy to show that

$$R = 1 - K_D \frac{1}{\left[\frac{S_T}{R} - nE_T\right]} \tag{2}$$

with K_D the dissociation constant, E_T the total enzyme concentration, and n the number of binding sites. This expression may be rearranged into

$$\frac{1}{1 - R} = \frac{1}{K_D}\frac{S_T}{R} - \frac{nE_T}{K_D} \tag{3}$$

Either expression allows us to determine K_D and n by measuring R as a function of the total ligand concentration S_T. Using eq 3, and plotting $1/(1 - R)$ vs. S_T/R, the slope of the straight line will give K_D and the intercept n. Using eq 2 and plotting R vs. $[(S_T/R) - nE_T]^{-1}$, a straight line will be obtained only for one integer value of n. The corresponding slope will give K_D. It is of course essential that the end value $\Delta\epsilon(\infty)$ is approached as closely as possible. The end value often cannot be reached in spectroscopic methods. It is no longer needed, however, when binding data are collected at two very different enzyme concentrations, since then the method of Halfman and Nishida (1972) can be applied. Another method to determine end values and dissociation constants from CD binding data was recently discussed (Greenfield et al., 1972).

The helix content of the enzyme was estimated using the methods of Greenfield and Fasman (1969) and of Chen et al. (1972).

Results

Protein CD Spectrum. The far-uv CD spectrum of the α subunit of tryptophan synthase is shown in Figure 1. The helix content was estimated using the ellipticity values at 208 and 222 nm. The resulting values of 46.4% from the method of Greenfield and Fasman (1969) and 29.0% from the method of Chen et al. (1972) do not agree very well

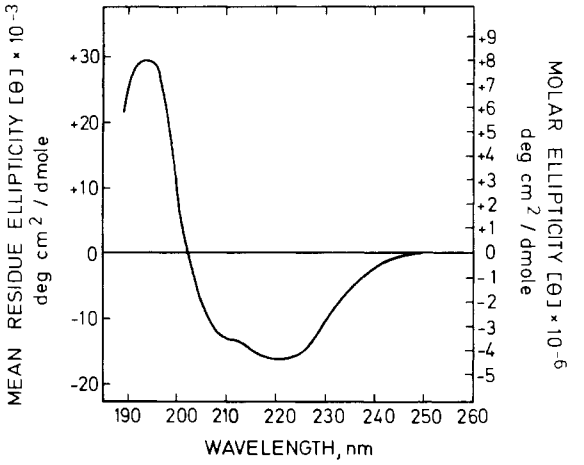

FIGURE 1: CD spectrum of the α subunit of tryptophan synthase in 5 × 10^{-2} M Tris-HCl buffer (pH 7.5) from 190 to 250 nm.

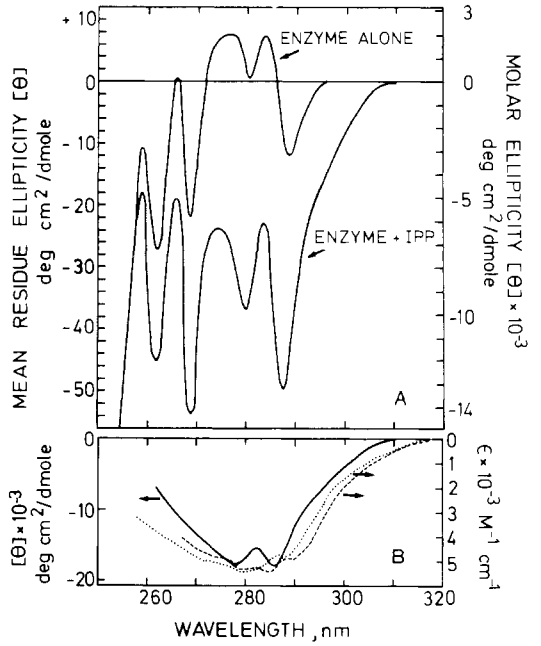

FIGURE 2: CD spectra of the α subunit of tryptophan synthase in 5 × 10^{-2} M Tris-HCl buffer (pH 7.5) from 250 to 320 nm. (A) Top curve, enzyme alone, concentration 51.5 μM; bottom curve, enzyme plus IPP, enzyme concentration 51.5 μM, IPP concentration 91 μM. (B) Comparison between the IPP-induced CD signal and the IPP absorption spectra; (—) difference between the CD spectra of the enzyme alone and the enzyme fully saturated with IPP; (· · ·) absorption spectrum of free IPP; (- - -) absorption spectrum of bound IPP.

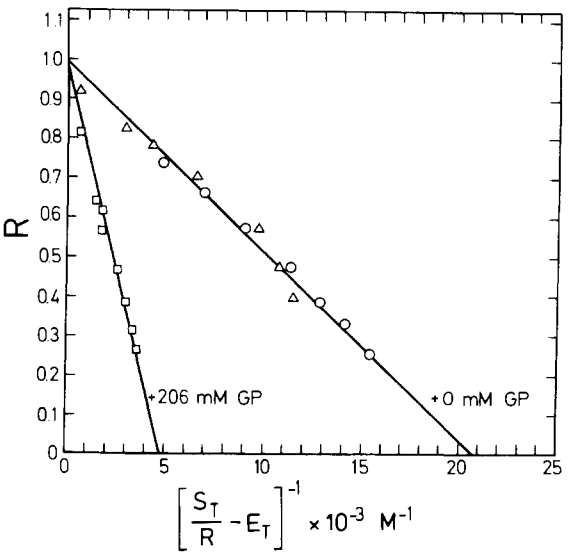

FIGURE 3: CD binding data for IPP plotted according to eq 2 with n = 1. S_T is the total IPP concentration. (△) Enzyme concentration 475 μM; (○) enzyme concentration 50.9 μM. The straight line corresponds to K_D = 48 μM. (□) IPP binding at enzyme concentration of 545 μM in the presence of 206 mM glycerol phosphate (GP). The straight line corresponds to $K_{D,eff}$ = 210 μM. The CD signal was recorded at 275 nm.

with each other. There can be little doubt, however, that the helix content is considerable, and much higher than the 16% obtained previously by optical rotatory dispersion (ORD) measurements (Freedberg and Hardman, 1971). Figure 2A shows the near-uv part of the CD spectrum. The characteristic fine structure of the 12 phenylalanine residues is well resolved in the wavelength region up to 270 nm. In addition to these, only the seven tyrosine residues make a contribution to the spectrum, since the enzyme contains no tryptophan and no disulfide bridges.

Binding of IPP. Unbound IPP and IND are optically inactive in the near-uv, whereas IGP shows a very small effect. The CD spectrum of the enzyme in this wavelength region in the presence of IPP is shown in Figure 2A. The CD difference spectrum is compared with the absorption spectrum of bound IPP in Figure 2B. Clearly the induced CD effect is entirely within the wavelength range where IPP absorbs and roughly proportional to the absorption spectrum. No perfect proportionality is expected, however, since the ^1L_a and ^1L_b bands of the indole chromophore, which are responsible for the absorption in this region, need not have the same dissymmetry factor $\Delta\epsilon/\epsilon$. As the induced spectrum overlaps with the protein spectrum, it cannot be excluded that the protein spectrum also changes upon binding. If substantial changes in the protein CD spectrum occurred upon IPP binding, no close similarity between CD and absorption spectrum such as in Figure 2B would be expected, however. Two arguments can be advanced to support this statement. The observed effect cannot be due to an internal tryptophan residue, since the enzyme does not contain tryptophan. In the second place it appears rather unlikely that the effect is due to the phenylalanine and tyrosine residues of the enzyme, because the difference spectrum has an appreciable amplitude at wavelengths above 300 nm. Additional evidence supporting our view that we are observing an almost pure extrinsic Cotton effect comes from the fact that IND and IGP show induced Cotton effects which are shifted to the blue with respect to the IPP CD spectrum in accordance with the corresponding shifts in their absorption spectra. The $\Delta\epsilon$ value for bound IPP at the CD maximum is 5.47 M^{-1} cm^{-1}, a value which is comparable to one recently found for the specific binding of another indole ligand to human serum albumin (Devaux et al., 1974).

The induced CD effect was used to follow the binding of IPP to the enzyme and was analyzed as described under Materials and Methods. The IPP binding data at 275 nm are plotted according to eq 2 in Figure 3. The experiments were performed at two different enzyme concentrations, 50.9 and 475 μM. Figure 3 shows that at both enzyme concentrations, we obtain n = 1 and K_D = 48 μM. When the data were plotted according to eq 3, straight lines were obtained with intercepts on the S_T/R axis equal to E_T, showing once again that only one binding site is present. These results are independent of the wavelength chosen to follow

the binding. At high enzyme concentrations, the denominator in eq 2 at low S_T values is a small difference between the two large numbers S_T/R and E_T. Slight changes in R, due for instance to inaccurate determination of the end value $\Delta\epsilon(\infty)$, result in large changes in the location of these points in a plot of eq 2. It is therefore important to work at sufficiently low enzyme concentrations.

In the far-uv a small change was observed in the CD signal at 222 nm when the enzyme was saturated with IPP. Free IPP has no CD signal at this wavelength. The amplitude at 222 nm decreases by about 5% in absolute magnitude. The effect was too small to carry out a titration experiment.

IPP Binding Enthalpy. The temperature dependence of the CD effect can in principle be used to determine the binding enthalpy. Since the pH of Tris buffer has a large temperature coefficient, the effect of pH on the CD spectra of the protein and the IPP-saturated protein was determined. No change could be detected in the relevant pH range from 7.1 to 8.2. With increasing temperature, the protein will eventually denature. Using the CD signal at 222 nm, it was ascertained that the native structure is preserved up to at least 45°. At fixed ligand concentration (about half-saturation), and at fixed wavelength, $R(T)$ was measured from 7.7 to 39.6°. At each temperature, the signals of the protein alone and of the fully liganded protein were recorded as well, since they are also slightly temperature dependent. Using eq 2, $K_D(T)$ was computed. A van't Hoff plot of these data resulted in a straight line. A least-squares fit led to a $\Delta H°$ value of -2.8 kcal/mol for the binding reaction.

IPP Binding to Denatured Enzyme. To see if the IPP binding is specific, requiring the intact secondary and tertiary structure of the protein, the enzyme was denatured in 6 M guanidinium chloride. In the near-uv CD spectrum of the denatured protein, the phenylalanine fine structure is not as well resolved as in the intact protein, and about 1 nm shifted to the blue. The contribution of the tyrosine residues to the spectrum, however, is considerably changed. It is now negative for all wavelengths and similar to that observed for the model compound *N*-acetyltyrosineamide in water (Shiraki, 1969). Addition of an amount of IPP, which would cause half-saturation when added to the native protein, produced no detectable change in the near-uv CD spectrum when added to the denatured protein. This leads to the conclusion that the binding of IPP requires the native structure of the enzyme.

Competition between Glycerol Phosphate and IPP. One may anticipate that the binding of IPP occurs via both its phosphate group and its indole group. An early indication that the phosphate group is involved was the fact that the IPP dissociation constant, determined by equilibrium dialysis, is 100 μM in 0.1 M phosphate buffer (Kirschner and Wiskocil, 1972) in contrast to a value of 48 μM in Tris buffer. In the presence of other phosphate-containing molecules, the IPP binding is expected to be reduced. The competition for the phosphate binding site of the enzyme between IPP and several phosphate containing analogs of its side chain was studied through its effect on the induced CD in IPP. All three analogs studied, D-GAP, glycerol phosphate (GP), and phosphoglyceric acid (PGA), when added to a solution containing IPP and enzyme, led to a clear reduction in the induced CD effect. Of these three compounds GP was selected for a quantitative study, since it contains no strongly reactive groups besides the phosphate group. The IPP CD binding experiment was repeated, as described above, in the presence of 0.2 M GP. Since the protein CD signal at 222 nm was unaffected by the high concentrations of GP used, we conclude that the protein is not denatured under these conditions. GP may of course also bind at other sites on the protein. What we are observing, however, is the specific effect of GP on the IPP binding. The results are plotted according to eq 2 in Figure 3. The same CD end value was reached at an appreciably larger IPP concentration. The induced CD spectrum has the same shape as in the absence of GP, indicating that IPP is bound in the same way as before. Again there is only one binding site, but the dissociation constant is increased to 210 μM. The simplest interpretation of our results is to assume that GP and IPP compete for the same phosphate binding site. The effective dissociation constant $K_{D,eff}$ determined by means of eq 2 is then equal to

$$K_{D,eff} = (1 + ([I]/K_I))K_D \quad (4)$$

with [I] the free inhibitor concentration, K_I the inhibitor dissociation constant, and K_D the IPP dissociation constant in the absence of GP. Substituting the measured values, a K_I of 60 mM is obtained. Interpreting the IPP binding constant of 100 μM in 0.1 M phosphate buffer in the same way, we calculate a K_I for the phosphate group of 90 mM. In spite of the large chemical difference between GP and the inorganic phosphate ion, the two inhibition constants are quite comparable. This may reflect the minor contribution of the organic side chain to the binding of GP. If binding through the phosphate group plays such a large role, then a ligand lacking this group, indolepropanol (IPL), should produce a much looser complex with a great reduction in the induced CD signal. Experimentally no induced CD signal was observed at an enzyme concentration of 0.5 mM, even at 1.7 mM IPL.

Fluorescence Titrations with IPP and IPL. A number of planar aromatic molecules show a blue shift in their fluorescence emission maximum when transferred from a polar medium to an apolar medium. This effect will occur in those cases in which the first singlet excited-state dipole moment is greater than that of the ground state. In the case of indole, this condition is fulfilled and the blue shift is well established. A table summarizing the blue shift data for indole in a number of solvents appeared recently (Weinryb and Steiner, 1971). An analogous transfer will probably occur in our case, since the hydrophobic indole group of IPP is expected to bind to some apolar region of the protein. Figure 4 shows that indeed a considerable blue shift occurs when IPP binds to the protein, clearly indicating that the indole part of the molecule is involved in the binding. IPP was excited at 305 nm, where the tyrosine absorption of the protein is negligible. The quantum yield seems to decrease upon binding. The effect is even more pronounced than it appears from the figure, since the extinction coefficient of bound IPP at 305 nm is larger than that of free IPP. In general the influence of solvent polarity on quantum yield is not well understood. For most indole derivatives a large increase in quantum yield is observed when they are transferred into an apolar medium (Cowgill, 1967a). The indole quantum yield is, on the other hand, sharply reduced by the presence of SH groups in the neighborhood of the indole ring (Cowgill, 1967b). Since two or three SH groups are believed to be near the active site (Hardman and Yanofsky, 1965), and IGP and IND affect the SH group reactivity (Hardman and Yanofsky, 1965; Freedberg and Hardman,

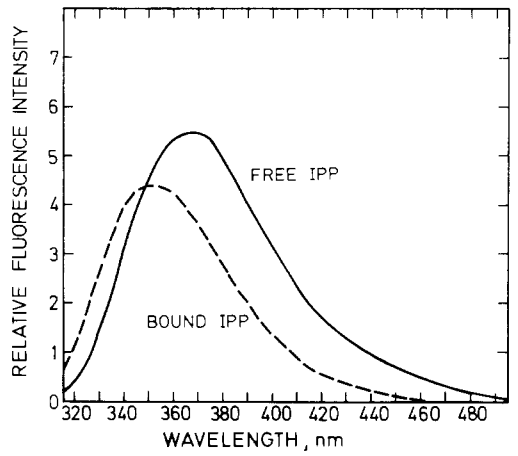

FIGURE 4: Effect of the α subunit of tryptophan synthase on the corrected fluorescence emission spectrum of IPP. (—) Free IPP (35 μM); (- - -) bound IPP (concentration scaled to 35 μM). The excitation wavelength was 305 nm. Enzyme concentration, 206 μM; temperature, 25°; 0.05 M Tris-HCl buffer (pH 7.5).

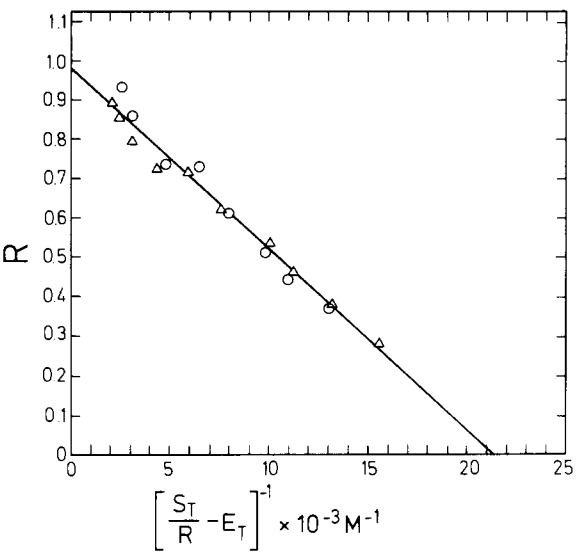

FIGURE 5: Fluorescence binding data for IPP plotted according to eq 2 with $n = 1$. (△) Enzyme concentration, 60.2 μM; (○) enzyme concentration, 51.6 μM. The straight line is a least-squares fit to all the data, resulting in $K_D = 46$ μM. Excitation at 305 nm, emission at 335 nm.

1971), this effect may well explain the otherwise anomalous drop in quantum yield.

The difference signal between free and bound IPP at 335 nm was used to perform a fluorescence titration experiment. The data were analyzed using eq 1–3. In this case the amount of bound IPP is proportional to the difference between the signal of IPP alone and the signal of IPP plus protein. The results are plotted in Figure 5 according to eq 2. From the slope and intercept we obtain $K_D = 46$ μM and $n = 1$, in excellent agreement with the CD results. The experiment was repeated with IPL instead of IPP under otherwise identical conditions (see Figure 4). No change could be detected in the IPL emission spectrum, indicating that at least in this concentration range no binding occurs.

IGP Binding. Since IGP is split into IND and D-GAP by the enzyme, a CD binding experiment with IGP can only be carried out after thermodynamic equilibrium has been reached. The equilibrium constant of the reaction is 4.4×10^{-4} M (Kirschner et al., 1975). At an enzyme concentration of 0.72 mM and an initial IGP concentration of 1.72 mM, the induced CD signal in IGP remained constant for at least 1.5 hr. Under these conditions, the equilibrium favors IGP synthesis. The induced CD signal with IGP is quite similar to that of IPP at the same concentration, except that the amplitude is somewhat smaller. Correcting for the small free IGP CD signal and using the reaction equilibrium constant above, the bound IGP CD spectrum can be calculated assuming that the binding constant for IGP is about the same as that of IPP. Comparing the induced spectrum with the bound IGP absorption spectrum, a great similarity, as in the case of IPP, is again observed. The induced CD signal of IGP is shifted to the blue with respect to the IPP CD spectrum, in accordance with the corresponding shift in the absorption spectrum. In view of the great sensitivity of the CD signal to binding geometry, the similarity in the induced CD signals may be considered as evidence that IPP and IGP bind in approximately the same way. This indicates that IPP is a good substrate analog.

IND Binding. Shortly after mixing with the enzyme, IND induces a positive CD effect in the near-uv, which lies in the wavelength range where IND absorbs as in the case of IPP and IGP. At a concentration of 1 mM, the amplitude of this signal is more than 25 times smaller than that of IPP. Over a period of hours, the originally positive IND signal changes, becoming negative for some wavelengths and remaining positive at other wavelengths. The amplitude remains very small. Possibly a very slow change in the protein conformation occurs. The binding of IND to the enzyme is known to be quite weak, with a dissociation constant of the order of several mM (Freedberg and Hardman, 1971). Because of the small induced CD effect, the large dissociation constant, and the large IND extinction coefficient, CD titration experiments could not be carried out, even with ultrathin cells (25 μm). In the far-uv spectrum, no detectable change could be observed even at a concentration of 9 mM IND. This finding is consistent with one of Freedberg and Hardman (1971), who observed no change in the ORD spectrum of the enzyme in the presence of 10 mM IND in the wavelength range 350–600 nm.

D-GAP Binding. The free ligand D-GAP shows an approximately Gaussian negative CD band centered at 290 nm. This band is most likely due to the $n \rightarrow \pi^*$ transition of the carbonyl group. A solution containing 0.6 mM enzyme and 12 mM D-GAP shows a CD spectrum which is not a simple superposition of the enzyme and the D-GAP spectra. The difference spectrum, which is obtained by assuming a free ligand spectrum for all the D-GAP present, consists of a positive band above 285 nm and a negative band below 285 nm. Since this difference spectrum is small with respect to the free D-GAP signal, a titration experiment could not be carried out. In the far-uv, no change in the protein spectrum could be observed up to a D-GAP concentration of 12 mM.

Discussion

The direct and indirect evidence favoring bifunctional binding of IPP and IGP can be summarized as follows. The extrinsic Cotton effect in the indole chromophore and the blue shift in the fluorescence emission maximum point to involvement of the indole group in the binding. The CD competition experiment between IPP and GP, and the reduction of the IPP CD signal in the presence of the phosphate-containing ligands D-GAP and PGA, clearly indicate

that the phosphate group participates in the binding as well. The dissociation constant in phosphate buffer is considerably higher than in Tris buffer. From steady-state kinetics of the IGP-synthesis reaction it is known that IPP is a competitive inhibitor also with respect to D-GAP. These facts are also consistent with the notion of a phosphate binding site on the enzyme. IPL, which lacks the phosphate group, but is otherwise identical with IPP, shows no blue shift in the fluorescence emission maximum and no induced CD effect up to m*M* concentrations. IND shows a barely measurable induced CD effect at m*M* concentrations. It seems that in the absence of the phosphate group the binding is much weaker or nonexistent. We are thus led to assume bifunctional binding of IPP to the enzyme. Because of the great similarity of the induced CD spectra of IPP and IGP, and the steady-state kinetic data showing that IPP is a competitive inhibitor with respect to IGP, it is quite probable that IGP binds bifunctionally as well. In addition to the direct evidence for involvement of the indole group, an indirect argument can be made against binding occurring exclusively via the phosphate group. In that case, the indole part of the molecule would have considerable rotational mobility. The effective average asymmetry seen by the chromophore would be greatly reduced in comparison with that in which the indole group is fixed. This mobility of the chromophore is thus expected to lead to a small CD effect. Conversely, the bifunctional binding will lead to a rigid ligand–enzyme complex, consistent with the large induced Cotton effect observed. These considerations are in agreement with the CD experiments of Chignell (1969) on the binding of phenylbutazones to human serum albumin. In this system there is also an electrostatic interaction with the protein, which is supplemented by van der Waals interactions between the phenyl groups and some hydrophobic area of the protein.

The bifunctional binding may be responsible for the increased thermal stability of the enzyme in the presence of IPP (Kirschner and Wiskocil, 1972), since IPP may link weakly two parts of the enzyme which are otherwise not directly coupled. Our findings suggest that further experiments in phosphate buffer will have to be corrected for IPP–buffer and IGP–buffer competition. The bifunctional binding mechanism and the competition with the phosphate buffer may also complicate chemical relaxation measurements.

The CD experiments with IPP show that we are most probably dealing with a pure extrinsic Cotton effect. It was shown that it is possible to determine a dissociation constant this way. The value of 48 μM from the CD experiment is in good agreement with the value of 46 μM from the fluorescence titration and the value of 48 μM from equilibrium dialysis (Weischet and Kirschner, to be published). One advantage of the CD method over equilibrium dialysis is its much greater speed. CD competition experiments and comparisons of CD spectra of substrates and inhibitors should also be of use with other systems.

The amplitude of the CD spectrum in the far-uv is a rough measure of the secondary structure of the enzyme. IND and D-GAP binding do not produce measurable changes in the CD spectra in this wavelength range. For IPP a small change was observed, which depended on the IPP concentration. Due to its small size it could not be titrated. It remains, therefore, an open question whether this change is simply an extrinsic Cotton effect induced by IPP binding, whether a ligand induced conformational change occurs, or whether both effects occur. A substantial amount of biochemical (Yanofsky and Crawford, 1972) and kinetic (Kirschner and Wiskocil, 1972; Kirschner et al., 1975) evidence exists for changes in the protein conformation upon binding of IND, IPP, IGP, and D-GAP. Our evidence is not necessarily in conflict with these experiments, since the sensitivity of CD in the far-uv with respect to small conformational changes is limited (Bayley, 1973). A small local change, mostly involving the side chains, may not be detectable. In the temperature jump kinetics of IPP binding to the α subunit of tryptophan synthase in Tris buffer, two relaxation times were observed. This led to the assumption of a ligand-induced shift in the equilibrium between two discrete protein conformations. Only if the two postulated states have large differences in secondary structure, and the binding preference for one form is sufficiently large, can we expect binding to produce a change in the CD spectrum. The small decrease observed when IPP binds may be due to this shift. At present it is experimentally impossible to distinguish between this explanation and the alternatives of a simple extrinsic Cotton effect, or a small conformational change brought about by the bifunctional binding of IPP. In view of the strong evidence in favor of a pure extrinsic Cotton effect in the near-uv, we are inclined to believe that in the far-uv we are observing a similar effect. The sum rule for rotational strength states that the net change in the CD spectrum upon IPP binding must be zero. The negative change in the near-uv must therefore be compensated for by positive changes in other parts of the spectrum. The observed change in the far-uv clearly overcompensates the negative near-uv change, however.

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Characterization of Nucleotide Binding Sites on Chloroplast Coupling Factor 1†

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ABSTRACT: A study of the equilibrium binding of ADP, 1,N^6-ethenoadenosine diphosphate, adenylyl imidodiphosphate, and 1,N^6-ethenoadenylyl imidodiphosphate to solubilized spinach chloroplast coupling factor 1 (CF_1) has been carried out. All four nucleotides were found to bind to two apparently identical "tight" sites, with characteristic dissociation contants generally less than 10 μM. The binding to these "tight" sites is similar in the presence of Mg^{2+} and Ca^{2+}, is stronger in 0.1 M NaCl than in 20 mM Tris-Cl, and is only slightly altered by heat activation. The slow rate of association of ADP and 1,N^6-ethenoadenosine diphosphate at these sites rules out the possibility that they are catalytic sites for ATPase activity on the solubilized enzyme. A third tight site for adenylyl imidodiphosphate was found on the heat-activated enzyme. The dissociation constant for this interaction (7.6 μM) is similar to the adenylyl imidodiphosphate competitive inhibition constant for ATPase activity (4 μM). ADP, which inhibits ATPase activity but is not a strong competitive inhibitor, binds only weakly at a third site (dissociation constant >70 μM). One mole of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole reacted per mole of CF_1 prevents ADP and adenylyl imidodiphosphate binding at the "catalytic" site and abolishes the ATPase activity. A model is proposed in which the "tight" nucleotide binding sites act as allosteric conformational switches for the ATPase activity of solubilized CF_1.

The coupling factor from spinach chloroplasts (CF_1)[1] is believed to be directly involved in photophosphorylation. The solubilized purified enzyme has no ATPase activity unless activated by trypsin (Vambutas and Racker, 1965), heat (Farron and Racker, 1970), or dithiothreitol (McCarty and Racker, 1968). While photophosphorylation and light induced ATPase activities in chloroplasts are dependent on Mg^{2+} (Petrack et al., 1961, 1965; McCarty and Racker, 1966), the activated CF_1 has a Ca^{2+} dependent ATPase, although a Mg^{2+} dependent ATPase may be induced by carboxylic acids (Nelson et al., 1972). The ATPase activity of CF_1 can be abolished by reacting NBD-Cl with one or two tyrosine groups on the β subunit (Deters et al., 1975), and the inhibition is completely reversed by dithiothreitol which releases the bound NBD.

In this work, equilibrium binding measurements are used to investigate the effects of heat activation, of Ca^{2+} and Mg^{2+}, and of NBD-Cl modification on the nucleotide binding sites of solubilized CF_1. These results are correlated with steady-state inhibition measurements of Ca^{2+}-ATPase activity by ADP and AMP-PNP. The solubilized CF_1 contains two tight binding noncatalytic sites for AMP-PNP, ϵAMP-PNP, ADP, and ϵADP, and the heat-activated enzyme contains an additional tight binding site for AMP-PNP, with the dissociation constant being essentially the same as the competitive inhibition binding constant. A mechanism is proposed for the allosteric control of ATPase activity in heat-activated CF_1.

Experimental Section

Materials. The ATP and ADP were purchased from Sigma Chemical Co. The AMP-PNP was obtained from P. L. Biochemicals and purified on a Dowex 2X-8 column using a 0–1.0 M ammonium formate gradient. The [^{3}H]ADP (5–15 Ci/mmol) and [^{3}H]AMP (5–15 Ci/mmol) were purchased from New England Nuclear. The [^{3}H]AMP-PNP (5–8 Ci/mmol) was obtained from I.C.N. and all the radioactive nucleotides were purified using paper chromatography with the solvent system isobutyric acid–1N ammonia (100:60 v/v). Imidodiphosphate was purchased from Boehringer Mannheim. The NBD-Cl was obtained from Pierce Chemical Company and the [^{3}H]NBD-Cl (8 Ci/mol) was a gift from Dr. D. Deters. All other chemicals were the best available commercial grade, and all solutions were prepared with deionized distilled water.

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[1] Abbreviations used are: CF_1, chloroplast coupling factor 1; F_1, mitochondrial coupling factor 1; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AMP-PNP, adenylyl imidodiphosphate; ϵAMP, 1,N^6-ethenoadenosine monophosphate; ϵADP, 1,N^6-ethenoadenosine diphosphate; ϵAMP-PNP, 1,N^6-ethenoadenylyl imidodiphosphate.