Cotton Effect Induced in Optically Inactive Molecules and Molecular Complexes by Optically Active Environment. III. Circular Dichroism Induced by Association of α-Chymotrypsin and Dyes

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Two types of circular dichroism (CD) spectra are found to be induced in the solution systems of α-chymotrypsin (αCT) and dyes. One is a single positive CD band which is due to a 1 : 1 αCT–dye complex (Type I; proflavine, Acridine Yellow G, acriflavine), and the other consists of one positive and one negative CD bands which are mainly due to a complex of the composition, one αCT to two dye molecules (Type II; Pyronin B, Rhodamine 6G). Mechanisms of CD induction tentatively proposed are that in Type I complexes a single dye molecule is bound to an asymmetric environment of the active site of αCT while in Type II complexes two dye molecules are bound at a specific site exerting a dipole-dipole interaction with one another.

There have been published a number of physicochemical and biochemical works on the interaction between biopolymers and dyes, of which the most elegant means may be the measurement of the Cotton effect induced in the corresponding visible absorption region of the dye. This is because once an accurate analysis is accomplished the bound dye molecules play a role as probes in determining the geometrical and electronic structures of the partner, biomacromolecules. Biopolymers that have been hitherto investigated in this way are nucleic acids,[1,2] polypeptides,[3] and polysuccharides,[4] and in all cases the primary cause of circular dichroism (CD) induction is recognized as a complex formation between polymer and dyes, though less quantitative description of such complexes has been found.

We have reported in a previous paper[5] that the Cotton effect is induced in the absorption region of a dye molecule embedded in cellulose diacetate film and proposed a mechanism of CD induction that the dyes are imprisoned in a cage formed by an assembly of the folded chains of cellulose diacetate and the Cotton effect is induced by an electrostatic interaction between electronic transition moments of the dye and of the chromophores involved in the cage. In the present paper, we set our eyes on a system of enzyme and dyes. The concept that an enzyme molecule forms an intermediate complex with a substrate or substrates in an early stage of enzymic action has been well established. It is also well known that each enzymic action has its specificity with respect to a single substrate. In the study of enzyme-dye interactions, whether some sort of specificity appears in a complex formation is an interesting subject. We measured CD spectra induced in a variety of combination systems of enzymes and dyes and tried to find out some interrelation between a way of grouping and whether and what sort of Cotton effects are induced in the visible absorption region of the dyes. We report in what follows the CD spectral features of α-chymotrypsin–dye solutions, the truth being that α-chymotrypsin was found to be the only enzyme to interact overnicely with some specific dyes.

Experimental

Commercial α-chymotrypsin, bovine pancreas (Nutritional Biochemicals, 3X crystallized and lyophilized) was purified by recrystallization three times. Proflavine, Acridine Yellow G, acriflavine, Pyronin B, and Rhodamine 6G were all commercial reagert grade and used without further purification. Stock aqueous solutions of α-chymotrypsin and the dyes were dissolved in 0.1 mol dm^{-3} Tris buffer (pH 8.1—8.5). In order to avoid aggregation of dye molecules, concentrations of the dyes were held in the range of $1—5\times 10^{-5}$ mol dm^{-3}. The molar concentration of α-chymotrypsin was calculated from its molecular weight which was assumed to be 25,000.

The absorption spectra were measured with a Hitachi model 124 spectrophotometer, the CD spectra by a JASCO J-40 polarimeter, and the optical rotatory dispersion (ORD) spectra by a JASCO ORD-UV-5 spectrophoto and polarimeter. All the measurements were performed at room temperature.

The ORD measurement (molecular rotation [ϕ]) had such a disadvantage that accurate determination became difficult when the concentration of α-chymotrysin was high, because the ordinanry dispersion of α-chymotrypsin extended over the absorption region of the dyes. For this reason, our primary attention was focused on the CD measurements. The molecular ellipticity [θ] of an induced CD (ICD) band was calculated by using the concentration of a dye involved.

Results

ICD spectra observed for the α-chymotrypsin (αCT)–dye systems may be divided into two classes; Type I consists of a single positive CD band, to which the αCT–proflavine (PF), Acridine Yellow G (AY), and acriflavine (AF) systems belong, and Type II is formed of a longer wavelength negative band and a shorter wavelength positive band, to which the αCT–Pyronin B (PB) and Rhodamine 6G (RG) systems belong. Since the ICD spectra of the same type (Types I and II) are not very much different from one another, we mention in detail only about the representative systems, αCT–PF and αCT–PB.

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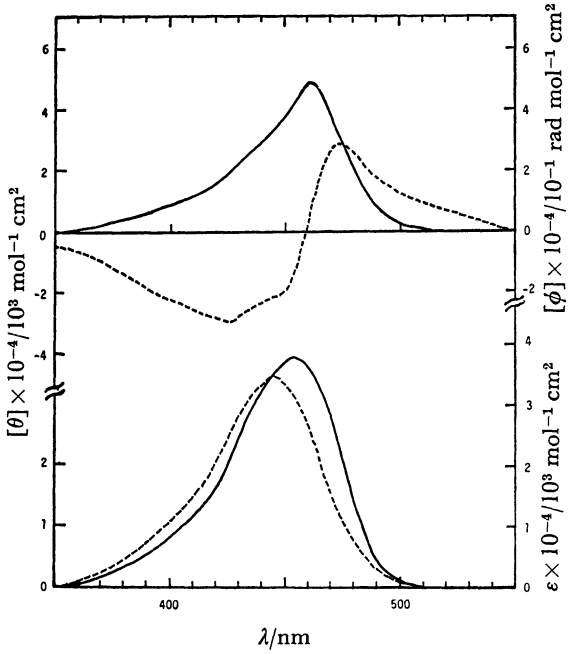

Fig. 1. CD (solid line, upper), ORD (broken line, upper), and visible absorption (solid line, lower) spectra of the α-chymotrypsin–proflavine complex dissolved in Tris buffer. $[D]=4.0\times10^{-5}$ mol dm^{-3} and [E]/[D]=3.8. The broken line in the lower half is the absorption spectrum of free proflavine of the same concentration.

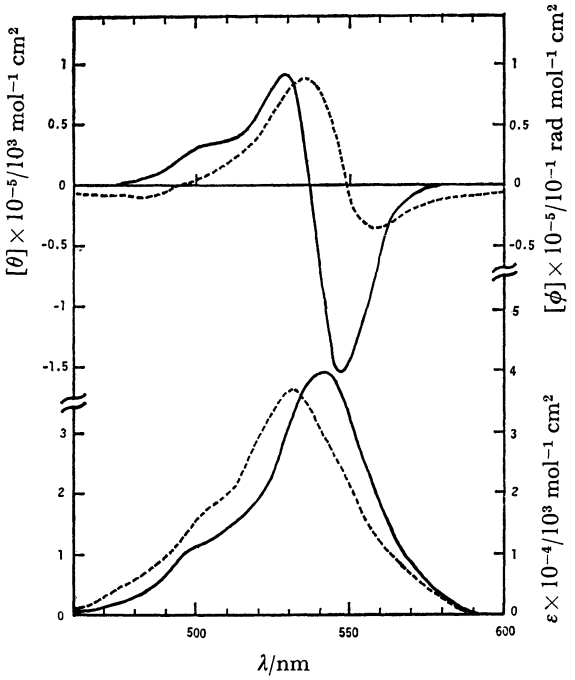

Fig. 3. CD (solid line, upper), ORD (broken line, upper), and visible absorption (solid line, lower) spectra of the α-chymotrypsin–Pyronin B complex dissolved in Tris buffer. $[D]=5.0\times10^{-5}$ mol dm^{-3} and [E]/[D] =4.0. The broken line in the lower half is the absorption spectrum of free Pyronin B of the same concentration.

α-Chymotrypsin–Proflavine. Figure 1 shows the CD, ORD, and visible absorption spectra of the αCT–PF system where the concentration of PF is 4.0×10^{-5} mol dm^{-3} and the molar concentration ratio of αCT to PF, [E]/[D], is 3.8. The single positive ICD band peaked at 462 nm corresponds to the broad absorption band of PF (peak: 456 nm; *cf.* peak of free PF: 445 nm), its molar ellipticity $[\theta]$ being 4.9×10^{4}. The corresponding ORD spectrum shows a positive Cotton effect. The peak intensity of the ICD spectrum was found to increase as [E]/[D] increases, while the peak wavelength and the spectral shape remained unchanged. Beyond [E]/[D]=8, the peak intensity itself remained constant, the corresponding $[\theta]$ value being 5.5×10^{4}. The relationship between $[\theta]$ and [E]/[D] is shown in Fig. 2.

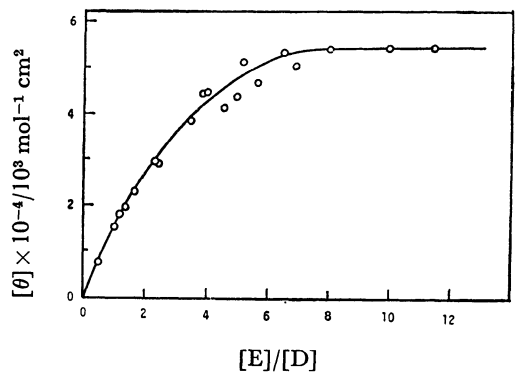

Fig. 2. Concentration dependence of the peak intensity of the induced CD band (462 nm) of the α-chymotrypsin–proflavine complex.

The absorption spectrum of PF shifts to the red and gains its intensity with the addition of αCT. The further shift of the absorption peak is not observed when [E]/[D]≧5, the peak converging to the value 460 nm. Furthermore, we found an isosbestic point at 443 nm.

α-Chymotrypsin–Pyronin B. Figure 3 illustrates the visible absorption and the induced CD and ORD spectra of the αCT–PB system, where the concentration of PB is 5.0×10^{-5} mol dm^{-3} and [E]/[D]=4. Contrary to the αCT–PF system, there appeared both positive and negative CD bands at the absorption region of the dye. The positive maximum lies at 529 nm with $[\theta]=9.1\times10^{4}$ and the negative maximum at 547 nm with $[\theta]=-1.54\times10^{5}$. Furthermore, a shoulder appeared at about 500 nm. As in the case of αCT–PF,

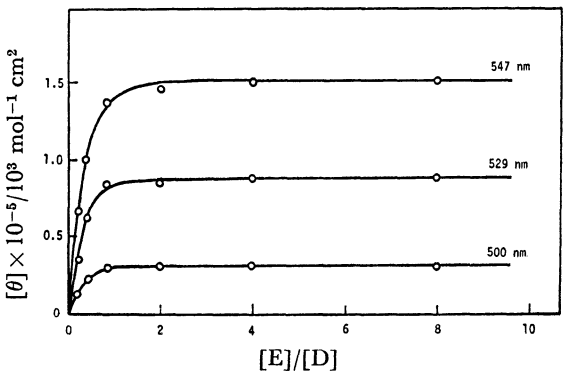

Fig. 4. Concentration dependence of the molecular ellipticities of the positive (529 nm), negative (547 nm), and shoulder (500 nm) CD bands of the α-chymotrypsin–Pyronin B complex.

the peak intensity was concentration dependent but the whole CD spectral feature remained unchanged. In Fig. 4 are plotted the molecular ellipticities of the positive, negative, and shoulder CD peaks against [E]/[D]. It is seen that in excess of [E]/[D]=4 the [θ] values tend to be concentration independent. The converged [θ] value for the positive band is 9.1×10^4, that for the negative band is -1.54×10^5, and that for the positive shoulder is 3.3×10^4.

The absorption spectrum of the αCT–PB system was quite similar to that of the αCT–PF system. The red shift of the absorption peak came to be over at 541 nm when [E]/[D]≧2. Contrary to the αCT–PF system, however, an isosbestic point was not observed explicitly.

The followings are brief descriptions of the results for the other systems.

α-Chymotrypsin–Acridine Yellow G. The positive ICD band was observed as in the case of αCT–PF. The peak of the CD band lies at 461 nm and its concentration dependence closely resembles the αCT–PF system; the [θ] value converges to 8.6×10^3 when [E]/[D]>4. The peak of the visible absortion spectrum of the system shifts from 435 nm (free dye) to 445 nm when [E]/[D] is varied, the latter being the converged value for [E]/[D]>4.

α-Chymotrypsin-Acriflavine. The spectral features of the ICD and visible absorption bands were just a copy of αCT–PF and/or αCT–AY.

α-Chymotrypsin–Rhodamine 6G. Like the αCT–PB system, the positive and negative ICD bands were observed. The whole spectral features of the CD bands are very much like those of αCT–PB, together with the similarity in the appearance of a weak shoulder in the shorter wavelength side. The corresponding ORD curve also resembles the case of αCT–PB. The molecular ellipticities of the negative (548 nm), the positive (529 nm), and the shoulder (505 nm) bands increase with the values of [E]/[D] and tend to converge to the value -5.3×10^5, 3.1×10^5, and 1.2×10^5, respectively, in excess of [E]/[D]=4. The visible absorption spectrum of αCT–RG behaves in the same fashion as that of αCT–PB upon concentration variation.

Finally, we like to add that no ICD spectra were observed with the solution systems of αCT and the following chromophores, though their molecular shapes are more or less analogous to those of the species described above: 9-aminoacridine, Methylene Blue, Toluidine Blue 0, thionine, Pyronin G, Rhodamine B, phenosafranine, Erythrosin B, Eosin B, Eosin Y, and Methyl Orange. Neither spectral shifts were observed in the visible absorption spectra of these solution systems.

Discussion

The observations described above for the αCT–specific dye systems clearly show a complex formation between αCT and the dyes. The appearance and the general features of the induced CD spectra in the aforementioned systems closely resemble those in the systems of nucleic acids, polypeptides, and polysuccharides with a variety of dye molecules.[1-4] Schlessinger and Steinberg[6] reported the appearance of CD absorption induced and circular polarization of fluorescence emitted in αCT–substrate complexes in which the substrates had been proven to bind at specific sites of αCT. However, the appearance of ICD in a system of an enzyme and a molecule which is understood not to receive specific enzymic actions has not been reported before.

As can be seen from the results described before, the ICD spectra and/or the modes of interaction of αCT and the dyes are discriminated into two classes without doubt. The following discussions will be divided into the Type I and Type II complexes.

Type I Complexes. From the shift of the visible absorption spectrum observed in the αCT–PF system, Bernhard *et al.*[7] concluded that a 1 : 1 complex is formed between αCT and PF and the binding site is the enzymic active site of αCT. The latter was deduced from the fact that PF behaves like a competitive inhibitor in enzymic reactions. Bearing in mind that the visible absorption spectrum of the αCT–PF complex resembles the spectrum of PF in various dioxane–water mixtures, a hydrophobic environment of αCT seems to be the primary cause for the formation of the complex. As can be seen in Fig. 2, the [θ] value increases with [E]/[D] until [E]/[D] ≈8 where almost all of the dye molecules are guessed to bind to αCT. The corresponding [θ] value for these saturated states, 5.5×10^4, is considered to be the molecular ellipticity for the αCT–PF complex. Moreover, it is possible that a specific binding site of PF is unique as evidenced from the fact that the shape of ICD spectrum remains unchanged with the increase of [E]/[D].

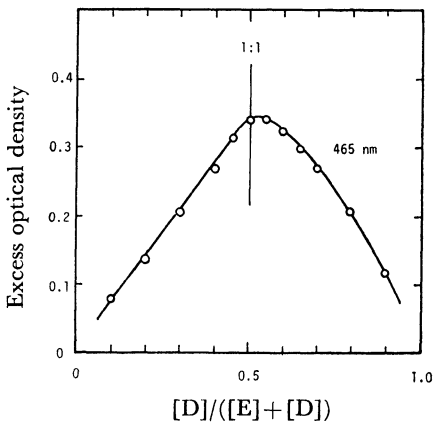

Fig. 5. Demonstration of formation of α-chymotrypsin–proflavine complex by method of continuous variations. [E]+[D]=2.0×10^{-4} mol dm^{-3}.

In order to find out a stoichiometric formula for the complex, we used the method of continuous variations.[8] In Fig. 5 is plotted the difference optical density at 465 nm as a function of [D]/([E]+[D]). From this, it is clear that one PF molecule binds to one αCT molecule in accordance with the conclusion made by Bernhard *et al.*[7]

In previous papers,[2] we proposed two mechanisms of CD induction in the system of DNA– and RNA–dye complexes; one which appears in high P/D (nucleic

acid phosphate to dye ratio) complexes is due to a static asymmetric perturbation caused in a dye monomer (referred to as optical activity I) and the other which appears in low P/D complexes results from an interaction between two dye molecules bound to the nucleic acid helices in the form of a dimer (optical activity II). In other words, the former arises from a one-electron mechanism in a perturbed single dye molecule and/or from an interaction between electric dipole transition moments of a single dye molecule and of neighboring chromophores involved in a macromolecule, and the latter from the exciton-type coupling mechanism between the two dye molecules bound to the biopolymer side by side. The general features of the single positive CD spectrum (Type I) of the αCT–PF complex and optical activity I above are not very much different from one another, so that the mechanism of CD induction in the αCT–PF complex is deduced to be a one-electron origin or the like. That is, αCT and PF form a 1 : 1 complex in which PF is bound near the asymmetric environment of αCT and CD is induced by the interaction of an electrically allowed transition of the dye with magnetically and electrically allowed transitions of the nearby asymmetric perturbers. The very same discussion will apply to the αCT–AY and αCT–AF systems, though we have not shown data of stoichiometric measurements for these complexes.

The questions still remaining are two fold; a definite site of the dyes binding in αCT and a reason why no CD spectra are induced in αCT and analogous dye systems mentioned before. These two problems are related to one another. First of all, it is certain that there is only one dye-binding site per one αCT molecule in the Type I complexes because the absorption spectral shift and the induced CD are like one another. Another basis for this deduction is that many other molecular homologues which have the acridine and phenothiazine rings do not form molecular complexes with αCT, as evidenced from the finding that there is no visible spectral shift in these systems. That is, the acridine and phenothiazine dyes can be classified into two as for the complex formation with αCT; one forms a 1 : 1 complex (Type I) and the other does not, the fashion of complex formation being unique and once a complex is formed CD being induced.

Secondly, it is quite possible that the site of dye binding is the enzyme active site of αCT, since that there exists only one substrate binding site per enzyme molecule has been well established with αCT. Bernhard *et al.*[7] demonstrated that monoacylation of αCT completely displaces the PF molecule from the enzyme active site. It seems therefore that the specific enzyme-dye interaction is restricted to the conformation of the enzyme active site. In fact, we found that no complexes were formed between the same dyes as studied in this paper and pepsin and/or papain.

As will be reported in a forthcoming paper,[9] a dye molecule has to be imprisoned in a certain segment of a partner macromolecule in order to induce optical activity. At the active site of αCT, there is a spatial blank of moderate size surrounded with Histidyl 57, Seryl 195, and Methionyl 192. Whether an enzyme–dye

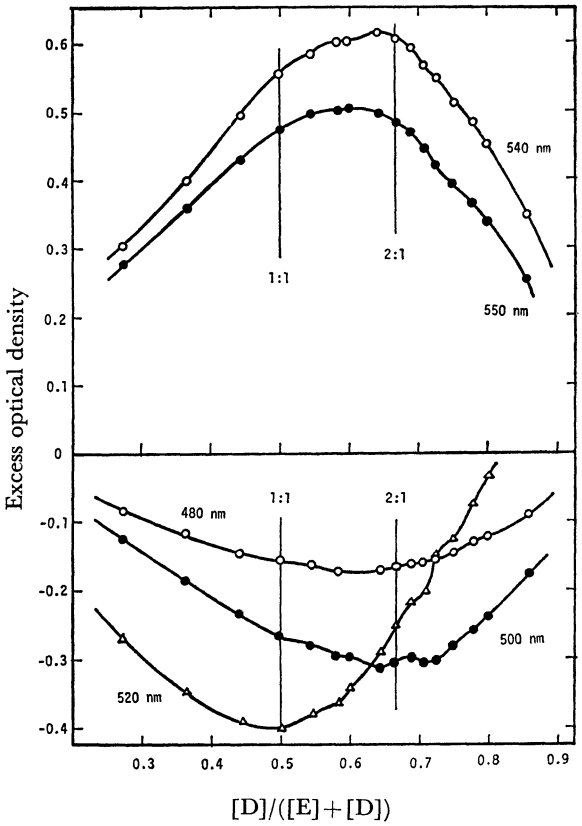

Fig. 6. Demonstration of formation of α-chymotrypsin–Pyronin B complex by method of continuous variations. $[E]+[D]=1.0\times10^{-4}$ mol dm^{-3}.

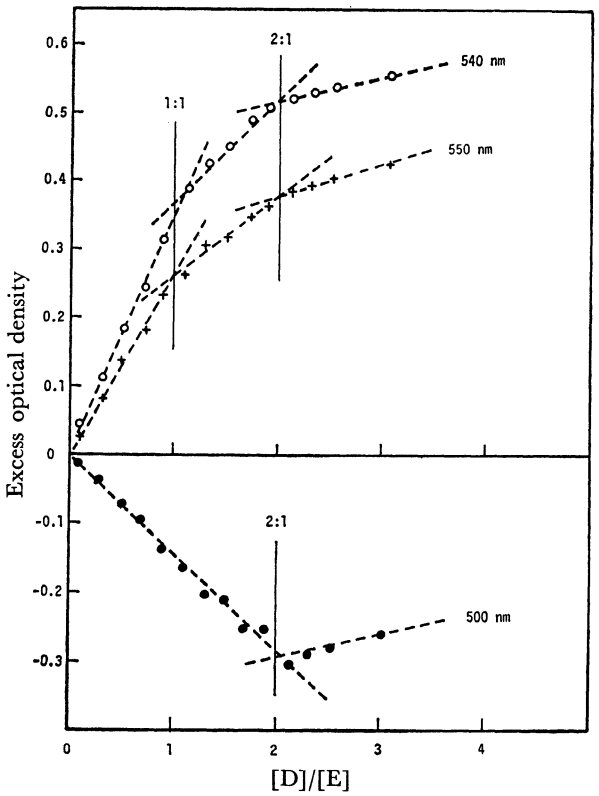

Fig. 7. Demonstration of formation of α-chymotrypsin–Pyronin B complex by molar ratio method. $[E]=3.6\times10^{-5}$ mol dm^{-3}.

complex is formed then depends upon a specific hydrophobic environment of the enzyme active site. This situation looks something like the well-known "lock-and-key" theory in the mechanism of enzyme action.

Type II Complexes. We will discuss the αCT–PB system as a Type II complex in which both positive and negative CD bands are induced. As in the case of the Type I complexes, the visible absorption spectrum of PB shifts to longer wavelength side upon addition of αCT, its general feature resembling closely to that of the absorption spectrum of PB brought about by the decrease of solvent polarity. It is clear therefore that the change in the visible spectral region of the αCT–PB system is due to the binding of PB to αCT which is less polar than water. The reason why a definite isosbestic point is not obtained is probably that there are more than two species of complexes in which stoichiometric compositions of αCT *vs.* PB are different from one another. The experiment of continuous variations revealed that there exists a complex formed by one αCT molecule and two PB molecules in addition to a 1 : 1 complex (Fig. 6). The same result was obtained by using the molar ratio method (Fig. 7).

It is hard to estimate the composition ratio of these two-type complexes. However, one can utilize the following experimental findings in order to guess whether 1 : 1 or 1 : 2 complexes is predominate to induce CD bands: (i) The variation of [E]/[D] with keeping [D] constant does not yield even slight change of the ICD spectral pattern; (ii) the molecular ellipticity becomes constant when [E]/[D] is in excess of 4 (*cf.* Fig. 4); the $[\theta]$ values of the Type II complexes are larger by an order of magnitude than those of the Type I complexes. We may thus conclude that the ICD spectrum shown in Fig. 3 is mainly due to the 1 : 2 complex.

When two dye molecules of the same species are brought together somewhere not far from one another, there occurs a rather strong dipole-dipole coupling. The leading term of the rotatory strength in this case is proportional to $\mathbf{R}\cdot(\boldsymbol{\mu}_1\times\boldsymbol{\mu}_2)$, $\boldsymbol{\mu}_1$ and $\boldsymbol{\mu}_2$ being the electric dipole transition moments of dye 1 and dye 2 respectively and $\mathbf{R}$ being the separation between $\boldsymbol{\mu}_1$ and $\boldsymbol{\mu}_2$.[10] If this is the case in the Type II complexes, the ICD spectrum may be resolved into four components, since in acridine dyes there exist two electric dipole transition moments, one along the molecular long axis and the other along the molecular short axis, both being in the molecular plane. We have carried out a Kronig-Kramers type analysis for the ICD spectrum and the observed optical rotatory dispersion curve of the αCT–PB system (Fig. 3). A resolution of the ICD bands is demonstrated in Fig. 8 which shows that two rotatory strength "couplets" are characteristic forms of dispersion. That is, the observed ICD spectrum is understood as a sum of the two Cotton effects of equal but opposite rotatory strength which are centered at 540 nm and lie less than a band width from one another, and of the other two Cotton effects of similar but smaller type centered at 520 nm. From the coincidence between the recomposed curve and the observed ICD spectrum (the recomposed and observed rotatory dispersion

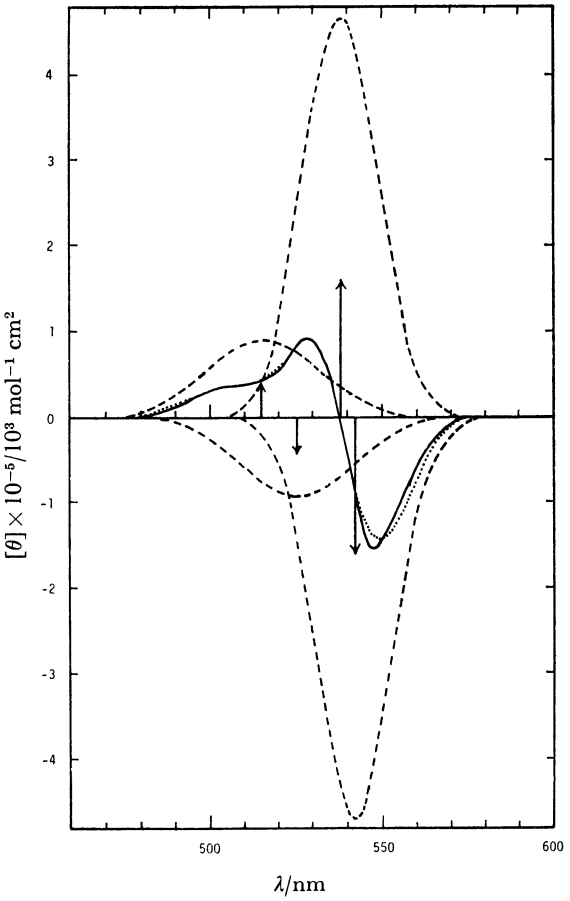

Fig. 8. Resolution of induced CD bands of α-chymotrypsin–Pyronin B complex assuming dipole-dipole coupling. The arrows show the positions and the strengths of electric dipole transition moments, the broken lines the component CD bands, the dotted line resultant CD bands, and the solid line observed CD bands.

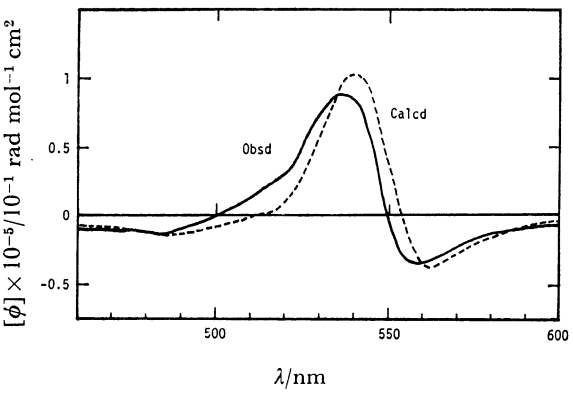

Fig. 9. Recomposed and observed rotatory dispersion curves of α-chymotrypsin–Pyronin B complex.

curves are compared in Fig. 9), a contribution from the 1 : 1 complex is unable to be taken into account. It is possible to speculate that the single dye molecule binds at the active site of αCT and the dye dimer binds at some other specific site, the contribution of the former to CD induction being negligibly small. On the other hand, our feeling at present is that a dye molecule

in the Type II complex is bound at a specific site which is not the active site of αCT and is therefore unable to act as a perturber for CD induction, the second dye molecule getting close to and keeping in a favorable direction side by side with the first dye molecule. If this is the case, the observations described above are well explained and it turns out that by some reason Pyronin G and/or Rhodamine B neither forms a complex with αCT nor associates one another in the neighborhood of the αCT molecule. No answer is possible to a question why there are two species of dyes, one forms a complex with αCT and the other does not.

Conclusion and Remark

The results and interpretation on the induced optical activity of the αCT–dye systems are summarized as follows:

(1) Some dyes form complexes with αCT with the appearance of CD and others do not.

(2) There are two ways of complex formation; whether a dye monomer is bound to αCT (Type I) or a dimer-like dye aggregate (Type II) is.

(3) To the Type I complex, proflavine, Acridine Yellow G, and acriflavine belong, yielding a single positive CD band. Composition of the complex is 1 : 1.

(4) To the Type II complex, Pyronin B and Rhodamine 6G belong, yielding one positive and one negative CD bands. Composition of the complex is mainly two dye molecules to one αCT.

(5) In the Type I complex, the site of dye binding is probably the enzyme active site of αCT which acts as an asymmetric perturber to induce CD in the visible absorption region of the dye.

(6) In the Type II complex, a dye molecule is bound at a specific site which is probably not the active site of αCT, and the second dye molecule combines with the first one in a fashion of forming a dimer. The coupling between the electric dipole transition moments of the two dye molecules is a leading term of the induced rotatory strength.

There are a lot of remaining questions: What is the reason for item (1)? Why any other enzyme does not form complexes with dyes such as those studied in this paper? Is there anything wrong with the reasoning for the site of dye binding? Experiments of the circular polarization of fluorescence and the fluorescence detected circular dichroism in the visible region are in progress, being aimed at the examination of the interaction mechanism in excited states of enzymes and dye chromophores.

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