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CHARACTERIZATION OF THE DIFFERENCE SPECTRUM OF DIISOPROPYLPHOSPHORYL-α-CHYMOTRYPSIN *VERSUS* α-CHYMOTRYPSIN

IV. THE ENVIRONMENT OF TRYPTOPHYL RESIDUES

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SUMMARY

The nature of the ultraviolet difference spectrum of diisopropylphosphoryl-α-chymotrypsin *versus* α-chymotrypsin is considered in this paper. A comparison was made of the difference spectrum of diisopropylphosphoryl-α-chymotrypsin *versus* chymotrypsin and the difference spectra of amino acid derivatives in media of varying polarity. The difference spectrum of acetyl-L-tryptophan amide in 20 % (v/v) glycerol *versus* this derivative in water (a more polar solvent) indicates that the spectra of tryptophyl residues, modified through incorporation into a protein, could easily account for the difference spectrum of diisopropylphosphoryl-α-chymotrypsin *versus* chymotrypsin. These observations suggest that one or more tryptophyl residues of diisopropylphosphoryl-α-chymotrypsin are in a less polar medium than the analogous residues in chymotrypsin.

Evidence against chemical substitution of a chromophore by the diisopropylphosphoryl group is given by the effect of urea denaturation on the diisopropylphosphoryl-α-chymotrypsin *versus* chymotrypsin difference spectrum. The solvent perturbation method of HERSKOVITS AND LASKOWSKI and the specific oxidation of tryptophyl residues in chymotrypsin and diisopropylphosphoryl-α-chymotrypsin by *N*-bromosuccinimide both indicate that the diisopropylphosphoryl-α-chymotrypsin *versus* chymotrypsin spectrum is caused by tryptophyl residues accessible neither to glycerol nor to *N*-bromosuccinimide. In conjunction with previous experiments, it can be concluded that the difference spectrum of diisopropylphosphoryl-α-chymotrypsin *versus* chymotrypsin is due to a non-ionic solvent effect and not to a charge or an inductive effect. Furthermore, the tryptophyl residues which give rise to the difference spectrum appear to be buried in both chymotrypsin and diisopropylphosphoryl-α-chymotrypsin.

Abbreviations: CT, α-chymotrypsin; DIP-CT, diisopropylphosphoryl-α-chymotrypsin; NBS, *N*-bromosuccinimide; ATEE, *N*-acetyl-L-tyrosine ethyl ester; ATrA, *N*-acetyl-L-tryptophan amide.

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INTRODUCTION

Earlier studies[1-4] demonstrated that the CT-catalyzed hydrolysis of *p*-nitrophenyl acetate is accompanied by absorbancy changes of the enzyme at 290 mμ which are related to the formation and decomposition of monoacetyl-CT. These absorbancy changes have also been observed[1-4] in the reaction of DFP with trypsin or CT. Evidence has been presented previously which indicates that the spectral changes at 290 mμ are due to conformational changes[3-8], brought about by the interaction of the substrate with the enzyme. In order to be able ultimately to identify the part of the CT molecule which participates in these reversible structural changes, initial investigations were concerned with the hydrogen ion equilibria of tyrosyl residues[9]. Reversible spectrophotometric titration experiments with CT and DIP-CT indicated that these residues are equivalent. This investigation is concerned with the environment of the tryptophyl residues in CT and DIP-CT.

MATERIALS AND METHODS

Materials

α-Chymotrypsin, three times crystallized, salt-free, obtained from Worthington Biochemical Corporation, was used without further purification. Assay of the lot, using the potentiometric method of SCHWERT *et al.*[10] and ATEE as the substrate at pH 8.0 and 15° in 10% (v/v) acetone gave a k_3 value of 77/sec. The value for CT reported in the literature[11] using the same substrate at 25° is: $k_3 = 115$/sec at pH 7.8 in 30% methanol.

DIP-CT was prepared by adding a 10–20-fold molar excess of 0.1 M DFP to chymotrypsin, 3 mg/ml, at pH 7.0.

Trypsin was obtained from Worthington Biochemical Corporation and pepsin from Nutritional Biochemical Corporation.

Reagents

Tris was obtained from Sigma. Calcium chloride, isopropanol, potassium chloride, sodium hydroxide, hydrogen peroxide, glycerol, sodium acetate, benzene, acetone, and urea, all analytical reagent grade, were from Mallinckrodt. *N*-Bromosuccinimide, acetonitrile, and maleic anhydride from Eastman Organic Chemicals were used without further purification. DFP, obtained from K & K Laboratories, Jamaica, N.Y., was diluted with anhydrous isopropanol, prepared by refluxing and distilling over Drierite. Sulfuric, hydrochloric, acetic, and phosphoric acids were "Baker Analyzed" reagents. ATrA and ATEE were from Mann Research Laboratories.

Instruments

A Cary Model 14 self-recording spectrophotometer was used for all difference spectra. A Beckman Model G pH meter was used for pH determinations, and measurements were made with reference to Beckman pH 4.0 and 7.0 standard buffers. For absorbancy readings, a Beckman DU spectrophotometer was used.

Protein concentration

Protein concentrations were determined spectrophotometrically at 280 mμ, using a molar extinction coefficient of 50000 (see ref. 12). The molecular weight of CT was taken as 25000 (see ref. 13).

The digestion of the proteins by proteolytic enzymes

5-ml aliquots of CT solution at pH 2.0 (about 2 mg/ml) were added to two 10-ml volumetric flasks. Crystalline pepsin was added to one solution to give a final pepsin concentration of 0.01 mg/ml. After 2 h at 37°, the solution was adjusted to pH 6.8 with 1 M potassium phosphate buffer, and crystalline trypsin was added to give a final concentration of 0.01 mg/ml. The digestion was continued for 40 min at 40°. The pH of this solution was then adjusted to 2.0 with HCl. The samples in experimental and control flasks were diluted to volume and the difference spectrum was recorded. Control experiments with trypsin and pepsin indicated that the contribution of the absorbancy of these enzymes to the difference spectrum was insignificant.

Nitrogen determination

The procedure described by JOHNSON[11] was followed. The digestion period was 3 h long.

Phosphorus determination[15]

5-ml samples containing between 12.5 and 100 mg of phosphorus were run in duplicate. The samples were digested over microburners with 2.2 ml perchloric acid and a boiling chip until almost colorless. A few drops of 30 % hydrogen peroxide were added and the samples were boiled until colorless and for an additional 10 min to destroy any residual peroxide. The remainder of the procedure was that followed by WOOTTON AND HESS[4].

Enzyme assays

Enzyme activity was measured using the method of SCHWERT *et al.*[10]. 8 ml of 0.005 M Tris–HCl buffer, 0.16 M in $CaCl_2$ (pH 8.0) and 4 ml of 0.05 M ATEE in 20 % (v/v) aqueous acetone were mixed by a mechanical stirrer in a water-jacketed sample cup at 15°. After the addition of 1 ml enzyme, the pH was kept constant by titrating with 0.2 N sodium hydroxide from a Gilmont Micropipet-Buret. The amount of base added was noted every 30 sec for 5 min.

N-Bromosuccinimide reaction

The samples were kept on ice. 2.2 moles of NBS are required to oxidize one mole of tryptophan in CT. Appropriate quantities of NBS were weighed into volumetric flasks, dissolved in 0.5 ml cold acetonitrile and diluted to volume with cold 0.2 M acetate buffer (pH 4.0). The NBS solution was slowly added to an equal volume of enzyme solution (9.2 mg/ml). The amount of tryptophan oxidized was calculated by the procedure of PATCHORNIK *et al.*[16]. After reaction with NBS, the samples for enzyme assays were dialyzed for 24 h at 4° in Visking 18/32 seamless cellulose tubing against several changes of 10^{-3} M HCl.

Urea denaturation

The procedure described by WOOTTON AND HESS[4] was followed.

Difference spectra

The procedure for obtaining the difference spectrum of ATEE in benzene *versus* ATEE in water will illustrate the use of the double-tandem cells of HERSKOVITS AND

LASKOWSKI[17]. Four solutions were prepared: 1-S, $3.6 \cdot 10^{-5}$ M ATEE in benzene; 2-S, water; 1-R, $3.6 \cdot 10^{-5}$ M ATEE in water; 2-R, benzene. The front half of the tandem cell in the sample compartment of the Cary spectrophotometer is filled with solution 1-S, the back half of the tandem cell with solution 2-S. The front half of the cell in the reference compartment contains solution 1-R, the back half contains 2-R. Before scanning each difference spectrum, the baseline of the instrument is adjusted to zero at 340 mμ.

RESULTS

Urea denaturation of CT and DIP-CT

To further clarify the interaction, if any, between the DIP-group and aromatic residues in the protein, denaturation studies were undertaken. In the experiments of WOOTTON AND HESS[4], the DIP-CT *versus* CT difference spectrum was maintained after denaturation in 9 M urea (pH 7.0). To determine if CT autolysis might account for this difference spectrum, analogous samples of CT were denatured by 9 M urea at pH 7.0 and 4.0 respectively. Then both solutions were adjusted to the same pH. No difference spectrum was observed. When CT and DIP-CT were denatured in 9 M urea at pH 4.0, no difference spectrum was detected, even when the solutions were subsequently adjusted to pH 7.0.

Experiments with model compounds

In previous papers[1-4] it was stated that the difference spectrum between DIP-CT and CT (Fig. 1a) is probably due to a non-ionic solvent effect. The nature of the difference spectrum suggested that one or more tryptophyl residues of CT become located in a less polar environment in DIP-CT. It became of interest, therefore, to investigate the medium effect on the spectra of appropriate model compounds. The difference spectra between solutions of unequal composition were obtained in double-tandem cells described by HERSKOVITS AND LASKOWSKI[17]. These cells allow subtraction of the solvent contribution to the difference spectrum.

The difference spectra at pH 2.6 of DIP-CT *versus* CT, ATrA in 20% (v/v) glycerol *versus* ATrA in water, and ATEE in 20% (v/v) glycerol *versus* ATEE in

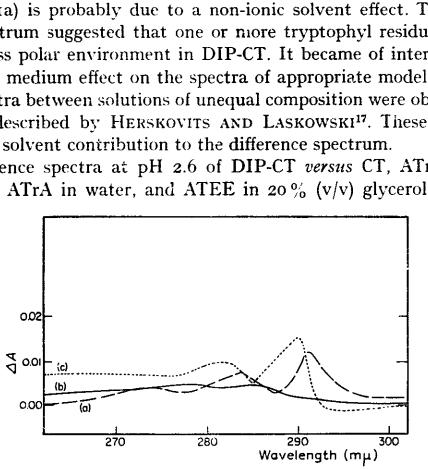

Fig. 1. Ultraviolet difference spectra. Curve a, ATrA in 20% (v/v) glycerol *versus* ATrA in water (pH 2.6) (citric acid); curve b, ATEE in 20% (v/v) glycerol *versus* ATEE in water (pH 2.6) (citric acid); curve c, DIP-CT *versus* CT (pH 3.8) (acetic acid–KCl) ($\mu = 0.17$). All compounds are $3.6 \cdot 10^{-5}$ M.

water are shown in Fig. 1. The concentration of the components is $3.6 \cdot 10^{-5}$ M in all cases.

Solvent perturbations of tryptophan spectra have sometimes revealed a small shoulder in the 270–280 mμ region[18,19]. The difference spectrum of DIP-CT *versus* CT does not have a clear shoulder near 275 mμ. Its similarity to the difference spectrum of ATrA in 20 % (v/v) glycerol *versus* ATrA in water is readily apparent (Fig. 1). The difference spectrum of ATEE in 20 % (v/v) glycerol *versus* ATEE in water is clearly different from the ATrA and the enzyme difference spectra.

When the perturbing solvent is benzene, a solvent resembling the nonpolar environment of buried residues in proteins more closely than 20 % glycerol, the results shown in Fig. 2 are obtained. The difference spectrum of CT *versus* partially digested CT is also shown in Fig. 2. CT contains four tyrosyl groups[20] but probably only two are exposed to solvent[9]. Since the molar absorbancy of tyrosine is small as compared to tryptophan, the difference spectrum of CT *versus* digested CT is essentially due to a difference in absorbancy between the buried tryptophyl residues in CT and the exposed tryptophyl residues in digested CT. The similarity between this difference spectrum (CT *versus* digested CT) and the difference spectrum of the ATrA in benzene *versus* ATrA in water is striking. It can be seen that the absorption maximum in the difference spectrum of ATrA has shifted only slightly in benzene as compared to 20 % (v/v) glycerol, while the absorption maximum of ATEE difference spectrum is at the same wavelength in benzene and in glycerol. The intensities of the maximum absorption peaks in the difference spectra, however, are considerably larger in benzene

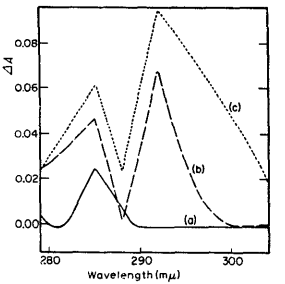

Fig. 2. Ultraviolet difference spectra. Curve a, ATEE in benzene *versus* ATEE in water, $3.6 \cdot 10^{-5}$M; curve b, ATrA in benzene *versus* ATrA in water, $3.6 \cdot 10^{-5}$ M; curve c, CT *versus* digested CT, $0.9 \cdot 10^{-5}$ M (pH 2.0) (HCl). The digestion of CT by pepsin and trypsin is described in the experimental section.

than they are in glycerol. The molar extinction difference coefficients ($\Delta\varepsilon_M$) for the maximum absorption peaks of the difference spectra and the wavelength at which the maximum is observed are listed in Table I.

Solvent perturbation

The solvent perturbation method of HERSKOVITS AND LASKOWSKI[17] was used. This method has been applied previously with excellent results to determine the

TABLE I

MOLAR EXTINCTION DIFFERENCE COEFFICIENTS FOR DIFFERENCE SPECTRA

Experimental conditions as described in Figs. 1 and 2.

Compound	*Perturbing solvent*	*Wavelength of maximum absorbancy difference (mμ)*	$\Delta\varepsilon_M$
DIP-CT *vs.* CT		290	600
CT *vs.* digested CT*		292	11 300
N-acetyl-L-tryptophan amide	Glycerol 20% (v/v)	291	300
	Benzene	292	1 900
N-acetyl-L-tyrosine ethyl ester	Glycerol 20% (v/v)	285	130
	Benzene	285	700

* This value was obtained at pH 2.0. Digested CT was prepared by successive reaction of CT with pepsin and trypsin as described in the experimental section. The effect of newly formed ionized end groups contributes to $\Delta\varepsilon_M$.

exposed and buried aromatic residues in a variety of proteins[17,21-24]. It can be seen in Fig. 3 that the difference spectrum of DIP-CT *versus* CT is essentially the same in water (curve a) as in 20% (v/v) glycerol (curve b). It should be noted that the absorbancy of curves a and b is the same at 292 mμ. The difference spectrum of DIP-CT in 20% (v/v) glycerol *versus* DIP-CT in water is also shown in Fig. 3, curve c. A large absorption peak at 292 mμ appears due to perturbation of the exposed tryptophyl residues in DIP-CT (see ref. 24). Furthermore, CT in 20% (v/v) glycerol *versus* CT in water produces a difference spectrum identical to that of DIP-CT described above.

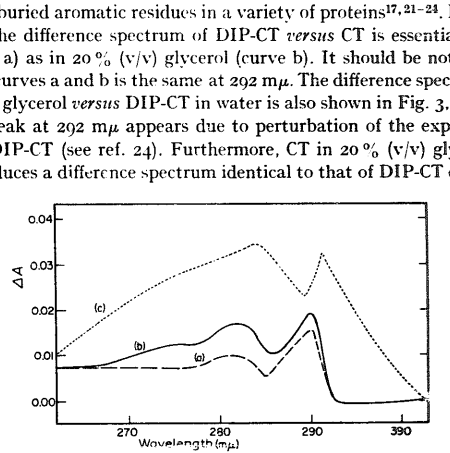

Fig. 3. Ultraviolet difference spectra at pH 3.8 (acetic acid–KCl) (μ = 0.17). Curve a, DIP-CT *versus* CT; curve b, DIP-CT *versus* CT, 20% (v/v) glycerol in both reference and sample cell; curve c, DIP-CT in 20% (v/v) glycerol *versus* DIP-CT in water. All compounds are $3.6 \cdot 10^{-5}$ M.

Oxidation of tryptophyl residues by N-bromosuccinimide

The reaction of NBS with tryptophan and tryptophyl residues in trypsin and CT has been investigated by WITKOP *et al.*[16,25,26]. These authors followed the reaction of this reagent in aqueous solutions of trypsinogen and trypsin by amino acid analysis and demonstrated that only tryptophyl residues were oxidized under their conditions[25]. In similar experiments with CT, VISWANATHA AND LAWSON[26] noted that in a sample of CT in which five tryptophyl residues were oxidized, one tyrosyl residue was also oxidized. The oxidation of tyrosyl residues leads to marked absorbancy changes at

260 mμ (see refs. 27, 28). In the experiments described below, absorbancy changes at 260 mμ were not observed.

The experiments of WOOTTON AND HESS[4] demonstrated that the tryptophyl residues of CT are more accessible to oxidation by NBS than the tryptophyl residues of DIP-CT. The aim of the experiments described below was to assign the origin of the difference spectrum to tryptophyl residues located either on the surface or in the inside of the molecule. A variety of group-specific reagents have been used previously to differentiate between exposed and buried amino acid residues of proteins[29,30]. CT was reacted with appropriate amounts of NBS at pH 4.0 to oxidize 1, 2, and 3 tryptophyl residues. Each oxidized sample was then divided into equal parts and a tenfold excess of DFP was added to one part and an appropriate amount of isopropanol was added to the other. The difference spectra of these oxidized CT solutions are shown in Fig. 4. Oxidation of one tryptophyl residue does not seem to affect the difference spectrum. The oxidation of two tryptophyl residues (curve a) decreases the magnitude of the difference spectrum appreciably, while in enzyme samples containing three oxidized tryptophyl residues (curve 2), the characteristic difference spectrum has almost disappeared.

The extent to which CT has become inactivated by the oxidation was then investigated. The results are tabulated in Table II.

In the course of the experiments it was observed that the oxidized enzyme fractions were all soluble at pH 4.0 where the oxidation was carried out. Protein fractions which contained more than two oxidized tryptophyl residues, however,

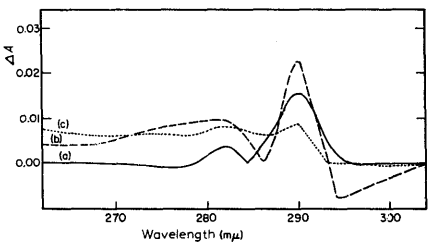

Fig. 4. Ultraviolet difference spectra. Curve a, DIP-CT *versus* CT, 2.4 tryptophyl residues oxidized in both proteins; curve b, DIP-CT *versus* CT, unoxidized; curve c, DIP-CT *versus* CT, 3.1 tryptophyl residues oxidized in both proteins. All samples are approx. $4 \cdot 10^{-5}$ M.

formed a precipitate at pH 7.0 which was removed by filtration. The material which remained in solution at pH 7.0 is called the soluble fraction. While no attempt was made here to further fractionate the material, the important point is that, regardless of the number of tryptophyl residues oxidized, the soluble fraction contains on the average two oxidized tryptophyl residues (Table II). CT with one tryptophyl residue destroyed can still be completely phosphorylated and has an observed maximum velocity about one-half of CT, using ATEE as the substrate. These findings are in agreement with observations[31,32] of CT in which one tryptophyl residue has been destroyed with methods other than NBS oxidation. As can be seen from Fig. 4, the difference spectrum of DIP-CT *versus* CT is decreased when about two tryptophyl

residues have been destroyed. However, in this sample the extent of phosphorylation has also decreased (Table II). The absorbancy change at 290 mμ in the difference spectrum of DIP-CT *versus* CT and the extent of phosphorylation appear parallel (Fig. 4, Table II).

TABLE II

EXTENT OF PHOSPHORYLATION AND ENZYME ACTIVITY OF OXIDIZED CHYMOTRYPSIN

*Tryptophyl residues oxidized**		*Moles of phosphorous per mole of enzyme*	*Enzyme activity*** *arbitrary units*
*Reaction mixture****	*Soluble fraction*§*	*Soluble fraction*§*	*Reaction mixture****
0	0	1.0	100
1.5	1.5	1.0	57
3.7	1.9	0.70	32
4.2	2.2	0.64	26

* Average number of tryptophyl residues oxidized as calculated by the method of PATCHORNICK, LAWSON AND WITKOP[16].

** Assayed with ATEE at pH 8.0 and 15°. A value of 100 corresponds to a k_3 value of 77/sec (see experimental section).

*** Includes pH 7.0-insoluble material.

§ Based on nitrogen determinations. A nitrogen value of 16.5% (see ref. 20) was taken for CT and a molecular weight of 25000 was assumed[13].

DISCUSSION

Experiments with model compounds suggest that at low and neutral pH, the peaks in the ultraviolet difference spectra of proteins between 275–287 mμ arise from perturbations of tyrosyl chromophores and those above 290 mμ from tryptophyl chromophores[33,34]. That the solvent perturbation of amino acids is a realistic model for observations made with proteins can be seen in Fig. 2, in which a comparison is made of the difference spectra of CT *versus* digested CT with the difference spectra of amino acid derivatives in benzene (simulating the internal environment of the protein) *versus* amino acid derivatives in water. The following interpretation of the data may then be made.

1. In previous experiments[1–4], we have reported that the difference spectrum of DIP-CT *versus* CT is stable when both enzymes are denatured by 9 M urea at pH 7.0, even when the pH of these solutions are subsequently changed. These experiments have been repeated and expanded. It was demonstrated that the DIP-CT *versus* CT difference spectrum was not due to autolysis of CT at pH 7.0, since analogous samples of CT, denatured by 9 M urea at pH 7.0 and 4.0 respectively and then adjusted to the same pH, gave no difference spectrum. When CT and DIP-CT are both denatured by 9 M urea at pH 4.0, a difference spectrum cannot be observed, even after pH adjustment to 7.0. Further experimentation is necessary to fully explain this interesting observation. The point to be emphasized is that under appropriate conditions, the difference spectrum of DIP-CT *versus* CT disappears, making a direct substitution of a chromophore by the DIP group extremely unlikely. Such a direct substitution was postulated[35].

2. The perturbation of ATrA by 20 % (v/v) glycerol gives a difference spectrum which closely resembles that of DIP-CT *versus* CT. Any dissimilarity can be explained by the incorporation of the tryptophyl chromophore into a protein. For example, a tryptophyl residue in a non-polar region in DIP-CT which is in a more polar region in CT could account for the observed difference spectrum. The small hump sometimes seen in pH and solvent difference spectra of tryptophan and its derivatives[18], is probably obscured by perturbation of other chromophores in the protein. CT has four tyrosyl residues[20]. As can be seen (Fig. 1) from the position and magnitude of the difference spectrum of ATEE in 20 % (v/v) glycerol *versus* ATEE in water, a change in environment around all the tyrosyl residues in DIP-CT could not account for the difference spectrum of DIP-CT *versus* CT. Even a medium effect more drastic than 20 % (v/v) glycerol (Fig. 2, curve a) does not appear to shift the peak of the tyrosine difference spectrum. In addition, it is known from spectrophotometric titration experiments[9], that the tyrosyl groups are equivalent in CT and DIP-CT; two titrate normally and two abnormally in both proteins.

3. Observations presented previously suggested that the difference spectrum of DIP-CT *versus* CT is not due to a direct perturbation of a chromophore by the DIP group[1]. The experiments recorded in Fig. 3 substantiate this point. It is shown that the difference spectrum of DIP-CT *versus* CT is the same in water as in 20 % (v/v) glycerol, while the difference spectrum of DIP-CT in 20 % (v/v) glycerol *versus* DIP-CT in water gives a peak at 292 mμ. The experiments of TANFORD *et al.*[36] indicate that the perturbing solvent used here does not change the conformation of the protein. If the difference spectrum of DIP-CT were due to direct interaction of the DIP group with tryptophyl residues, one would expect that these residues should also be accessible to glycerol. In this case the difference spectrum of DIP-CT *versus* CT in 20 % (v/v) glycerol would be different from the difference spectrum of DIP-CT *versus* CT in water. This was not observed. That a direct interaction of the DIP group with tryptophyl residues is unlikely is also demonstrated in the experiments discussed below.

4. The oxidation experiments show that two tryptophyl residues of CT are easily accessible to NBS and that oxidation of additional residues results in precipitation of the protein. In combination with the enzyme assays, it can be seen from Fig. 4 and Table II that the magnitude of the difference spectrum decreases proportionally to the enzyme activity. The measured activity of CT appears to be associated with a molecule which contains at most two oxidized tryptophyl residues*. NBS oxidation indicates that neither of the two tryptophyl residues readily reacting with NBS is involved in the difference spectrum of DIP-CT *versus* CT. The observation that the difference spectrum is the same in water as it is in 20 % (v/v) glycerol supports this. One must conclude, therefore, that the DIP-CT *versus* CT difference spectrum is due to tryptophyl residues inaccessible to NBS and glycerol and that the internal environment of these residues is different in CT and in DIP-CT. The possibility that an interaction between the DIP group and an internal tryptophyl residue occurs as a

* VISWANATHA AND LAWSON[26] in a paper on the oxidation of tryptophyl residues of CT by NBS, present data indicating that CT samples, containing five oxidized tryptophyl residues, still possess about 20 % of their original activity and are phosphorylated to an extent of 60%. The data presented in Table II suggest that the properties of oxidized CT observed by VISWANATHA AND LAWSON are due to a molecule in the reaction mixture in which only two tryptophyl residues have been oxidized.

result of conformational changes is not excluded, but a direct perturbation of tryptophyl residues by the DIP group is improbable.

5. The experimental data in Fig. 2 and Table I indicate that the difference spectrum of DIP-CT *versus* CT does not arise from absorbancy differences between tryptophyl residues completely buried in DIP-CT and residues completely exposed to solvent in CT. It should be noted that the peaks of the difference spectra shift to longer wavelength with increasing differences in the polarity of the perturbing solvent. The difference spectral peak of DIP-CT *versus* CT is at 290 mμ, that of ATrA in 20 % (v/v) glycerol *versus* ATrA in water is at 291 mμ, while ATrA in benzene *versus* ATrA in water produces a peak at 292 mμ.

Using the solvent perturbation method[17], WILLIAMS AND LASKOWSKI[21] reported that three of the tryptophyl residues of CT are exposed and four are buried. On this basis, the molar extinction difference coefficient ($\Delta\varepsilon_M$) for the perturbation of one tryptophyl residue by the internal environment of CT is at most 2800 (Table I), for the solvent perturbation by benzene 1900, and for the perturbation by 20 % (v/v) glycerol, 300. These values appear to be directly related to the difference in polarity of the environment of the perturbing media. The $\Delta\varepsilon_M$ value for the difference spectrum of DIP-CT *versus* CT is 600. Assuming that DIP-CT consists of a single compound, both the $\Delta\varepsilon_M$ value and the position of the peak of the difference spectrum indicate that media polarity differences causing the spectrum are small. This is consistent with the experiments discussed in Sections 3 and 4 which suggest that the tryptophyl residues which are responsible for the difference spectrum of DIP-CT *versus* CT are buried inside both molecules. It must be remembered, however, that the aromatic residues of CT were found to be more accessible to chemical reagents than the corresponding residues of DIP-CT (see ref. 4). In view of the experiments presented here, these observations most probably reflect the greater stability of DIP-CT as compared to CT under the conditions of the experiments rather than the reactivity of the aromatic residues *per se*. The increased stability towards denaturation of DIP-CT has been substantiated by a number of observations[4–8].

The data presented, together with previous experiments, show that the difference spectrum of DIP-CT *versus* CT is not due to a charge effect or to an inductive effect but to a non-ionic solvent effect on the tryptophyl residues. The residues giving rise to the difference spectrum are probably buried in DIP-CT and in CT.

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