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Low pH Dimerization of Chymotrypsin in Solution†

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ABSTRACT: The mechanism of the acid dimerization of α-chymotrypsin in solution was reexamined using a number of chemical derivatives. Blocking of the carboxyl of Tyr-146, while that of Asp-64 remained free, eliminated completely the ability of α-chymotrypsin to dimerize, as did methylation of His-57. O-Acetylation of Tyr-146 reduced the dimerization constant to that of γ-chymotrypsin, and deacetylation did not reverse it to the value of α-chymotrypsin. Acetylation and deacetylation of the other accessible tyrosines did not affect the dimerization. It is concluded that the mechanism proposed by Aune and Timasheff [Aune, K. C., and Timasheff, S. N. (1971) *Biochemistry 10,* 1609–1617] for the solution dimerization which involves the electrostatic interaction between the His-57 imidazolium ring and the terminal carboxyl of Tyr-146 is still most consistent with all the experimental observations. The interactions in dilute solution may differ somewhat from those observed in crystals. In particular, the two intermolecular bridges formed by sulfate ions in crystals cannot be present in solution.

The self-association in solution of chymotrypsin has been broadly investigated over the past 20 years. Below pH 5, α-chymotrypsin has been shown to exist as a monomer–dimer system in equilibrium, with a pH optimum of 4.1–4.3 (Egan et al., 1957; Timasheff, 1969; Aune and Timasheff, 1971; Aune et al., 1971; Horbett and Teller, 1973, 1974; Neet and Brydon, 1970). A thermodynamic analysis of the pH profile of this dimerization, together with an examination of the intersubunit contacts in the crystallographic dimer (Sigler et al., 1968; Birktoft et al., 1969), led Aune and Timasheff (1971) to conclude that the pH dependence was determined most probably by the formation of a pair of salt bridges between ionizable groups on the protein surface, the most likely groups being the imidazolium of histidine-57 and the carboxylate of tyrosine-146.

More recent, refined analyses of the contacts between ionizable groups in the dimer in the crystal state (Birktoft and Blow, 1972) have revealed the presence of other contacts and have questioned the validity of the interactions identified by Aune and Timasheff (1971) in the solution dimer. In a very detailed and elegant analysis of the crystal structure, Vandlen and Tulinski (1973) have found that, in the crystal, the contact in the dimer interface region across dyad A between Tyr-146 of one molecule and His-57 of the other molecule is either by the formation of a salt bridge between the carboxylate of Tyr-146 and the imidazolium ring of His-57 or by hydrogen bonding between the protonated carboxyl of Tyr-146 and the carbonyl of His-57, the second mode of interaction being more likely below pH 6. Another major interaction in the crystal state is the formation of an ion pair between the carboxylate of Asp-64 of one molecule and the α-amino of Ala-149 of the other molecule, a second pair being possibly formed in symmetrical fashion. In the crystal, an important source of dimer stabilization is afforded by two identical bridges formed by sulfate ions, each bridge being between the phenolic hydroxyl of Tyr-146 of one molecule and the hydroxyl of Ser-195 and the imino of Gly-193 of the other molecule (Vandlen and Tulinski, 1973). In solution, addition of sulfate ions weakens the dimer formation (Aune et al., 1971), which is contrary to what should be expected if a sulfate ion bridge were involved in this interaction. In view of these apparent contradictions between the crystal and solution results, further solution studies were undertaken using derivatives of chymotrypsin which affect the residues in question, and the results are reported in this paper.

Materials and Methods

Materials. α-Chymotrypsin, three times crystallized (lots OLC, CDI, 6084-5, 6102-3, 8JA, 8LK, 36J835, and 345888),

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γ-chymotrypsin, two times crystallized (lots 6204-5 and 6207), and chymotrypsinogen A, three times crystallized (lot CG 7FB), were purchased from Worthington Biochemical Corp. DIP-α-Ct[1] was from Worthington Biochemical Corp. (lot CDDFP 9CA) and from Sigma Chemical Co. (lot 123C-8051-9, three times crystallized). *N*-Acetylimidazole, methyl *p*-nitrobenzenesulfonate, hydroxylamine hydrochloride, semicarbazide hydrochloride, and trichloromethanesulfonyl chloride were from Eastman Kodak Co.; aminomethanesulfonic acid, lot 031847, was from Aldrich Chemical Co., Inc.; *N*-benzoyl-L-tyrosine ethyl ester was from Nutritional Biochemicals Corp.; CM-Sephadex C-50 was from Pharmacia Fine Chemicals; *N*-formyl-L-phenylalanine, Lot N-1795, was from Vega-Fox Biochemicals; L-1-tosylamido-2-phenylethyl chloromethyl ketone, lot 41C-0440, was from Sigma Chemical Co. *N*-Acetylimidazole was recrystallized twice from dry benzene prior to use and stored over P_2O_5. Semicarbazide hydrochloride was recrystallized from an alcohol–water mixture. α-Chymotrypsin was purified by chromatography on a CM-Sephadex C-50 column according to Nakagawa and Bender (1970) or on hydroxylapatite at pH 6.8 in phosphate buffer. DIP-α-Ct was purified on hydroxylapatite.

Preparation of Protein Derivatives. Destyrosine-146-α-chymotrypsin was prepared from the α enzyme by the method of Gladner and Neurath (1953, 1954) with a 100:1 chymotrypsin to carboxypeptidase ratio for large preparations (1 g and more), and a 10:1 ratio for micropreparations (50 mg of chymotrypsin). In the large preparations, the destyrosine-146 derivative was purified on a CM-Sephadex C-50 column according to Nakagawa and Bender (1970). The final step, precipitation with 0.7 M $(NH_4)_2SO_4$, could not be carried out, since the derivative did not precipitate under these conditions. The micropreparations of destyrosine-146-α-chymotrypsin were purified on hydroxylapatite columns using pH 6.8 phosphate buffer or $CaCl_2$ solutions for elution.[2] Methylhistidine-57-α-chymotrypsin was prepared according to Nakagawa and Bender (1970); Met-192-sulfoxide-α-chymotrypsin according to Taylor et al (1973) using trichloromethanesulfonyl chloride as oxidizing agent; TPCK-α-Ct[1] according to Shaw (1967); tosyl-α-Ct according to Kallos and Rizok (1963); and PMS-α-Ct according to Gold (1967). These five derivatives were purified by chromatography on hydroxylapatite at pH 6.8 in phosphate buffer. The blocking of carboxylic residues was carried out either using the procedure of Fersht and Sperling (1973) at pH 3.7 when the reagent was semicarbazide or that of Lin and Koshland (1969) at pH 4.0 when it was aminomethanesulfonic acid. A sample of chymotrypsin in which the carboxyls had been blocked with semicarbazide has also been generously given to us by Dr. A. Fersht. The carboxyl-blocked chymotrypsinogen was purified by chromatography on CM-cellulose (Rovery, 1967). It was activated under conditions of both slow and rapid activation (Wilcox, 1970; Fersht and Sperling, 1973). The product of slow activation was purified by two methods: chromatography on CM-cellulose at pH 6.0 in citrate buffer (Rovery, 1967) and on hydroxylapatite at pH 6.8 in phosphate buffer. Acetylation with *N*-acetylimidazole was carried out at pH 7.5 in 0.05 M borate buffer (Riordan et al., 1965) at 4 or 25 °C. The acetylated samples were dialyzed overnight at 4 °C in the appropriate buffer to remove excess reagent. The number of tyrosyl residues O-acetylated was calculated according to Riordan and Vallee (1967). The deacetylation was carried out by mixing equal volumes of acetylated protein and 2 M hydroxylamine at pH 7.0–7.5 (Hestrin, 1949). The number of total labile acetyl residues was determined spectrophotometrically according to standard procedures (Hestrin, 1949; Balls and Wood, 1956).

Removal of Tyrosine-146 from Di- and Tri-O-acetylated α-Chymotrypsin. Samples of di- and tri-O-acetylated α-chymotrypsin were treated with carboxypeptidase A–DFP at a 10:1 ratio in a 0.1 M pH 7.5 phosphate buffer containing ~3% LiCl for 3 h at 25 °C (Gladner and Neurath, 1953; 1954). The pH was then adjusted to approximately 3, and each solution was put to dialyze overnight at 4 °C in water at pH 4.3. After dialysis each solution was lyophilized. The extent of acetylation was again determined as described above (Riordan and Vallee, 1967). Each sample was then titrated in 1 N NaOH to pH 13.5 to determine the total number of tyrosines remaining. In these experiments, the reference cell contained chymotrypsin which had been acetylated, then exposed to pH 11.2–12.2 for 20 min to deacetylate the tyrosine residues, and finally adjusted to pH 3.

Enzyme Assays. The enzymatic activities of the chymotrypsins and derivatives were determined spectrophotometrically at 256.0 nm using *N*-benzoyl-L-tyrosine ethyl ester according to the method of Hummel (1959).

Spectroscopic measurements were done at room temperature on a Cary Model 14 recording spectrophotometer. The pHs were measured at room temperature with Radiometer Model 4 and TTTlc pH meters. Protein concentrations were determined spectrophotometrically in 0.001 M HCl. The absorptivity values used were: 20.0 dL/cm-g at 282 nm for chymotrypsinogen (Wilcox et al., 1957); 20.0 dL/cm-g at 280 nm for α-chymotrypsin (Marini and Wunsch, 1963), Me-α-Ct, DIP-α-Ct, Met-192-sulfoxide-α-chymotrypsin, TPCK-α-Ct, tosyl-α-Ct, and PMS-α-Ct; 20.1 dL/cm-g at 280 nm for γ-chymotrypsin (Gorbunoff, 1971); and 19.8 dL/cm-g at 280 nm for destyrosine-146-α-chymotrypsin. The last value was determined using concentrations obtained from dry-weight measurements of the purified protein which had been dialyzed overnight against 0.001 M HCl. The molecular weights were taken as 25 245 for the α-, γ-, and Me-α-Ct and as 25 083 for the destyrosine-chymotrypsins, based on their amino acid compositions (Dayhoff and Eck, 1967). The molecular weight of chymotrypsinogen was taken as 25 000. Monomer molecular weights of other chymotrypsin derivatives were obtained by taking into account the contribution of substituent groups.

Spectrophotometric titrations were carried out by the difference spectrophotometric technique (Wetlaufer, 1963) in 0.5 and 1 M NaOH, the reference solution consisting of protein dissolved in 0.001 M HCl. The increase in optical density in the 295–300-nm range was followed for 1 h, although the maximal value of the optical density was attained within 15 min. The number of ionized tyrosine residues was calculated by the previously described procedure (Gorbunoff, 1971). The titration of chymotrypsin derivatives from which Tyr-146 had been removed was accompanied by the titrations of the starting material and of an α-chymotrypsin reference sample which had been subjected to the identical carboxypeptidase treatment.

Sedimentation Equilibrium. Samples for sedimentation equilibrium were dialyzed for 24 h at 6 °C vs. a large excess of buffer. After dialysis, the concentration was adjusted, if necessary, with the dialysis buffer. The sedimentation equilibrium experiments were carried out by the meniscus-depletion method of Yphantis (1964) at 24 000 rpm and 25 °C in a Beckman Model E analytical ultracentrifuge equipped with

[1] Abbreviations used: α-Ct, α-chymotrypsin; Ctgen, chymotrypsinogen; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; PMS-α-ct, phenylmethanesulfonyl-α-chymotrypsin; DIP-α-Ct, diisopropylphosphoryl-α-chymotrypsin; BTEE, *N*-benzoyl-L-tyrosine ethyl ester.

[2] M. J. Gorbunoff, to be published.

TABLE I: Acid Dimerization of α-Chymotryspin and Derivatives.[a]

sample	pH	K (L/mol)	$-\Delta G°$ (kcal/mol)
α-chymotrypsin	4.12	2.6×10^4	6.0
MeHis-57-α-Ct	4.12	~0	
TPCK-α-Ct	4.12	~0	
tosyl-α-Ct	4.12	2.7×10^3	4.7
PMS-α-Ct	4.12	2.4×10^3	4.6
DIP-α-Ct	4.12	1.5×10^3	4.3
α-Ct-semicarbazide	4.12	~0	
α-Ct-amidomethanesulfonic acid	4.12	~0	
act. prod of Ctgen-semicarbazide	4.12	~0	
des-Tyr-146-α-Ct	4.12	~0	
γ-chymotrypsin	4.12	4.8×10^3	5.0
α-chymotrypsin	4.70	1.4×10^4	5.7
γ-chymotrypsin	4.70	3.1×10^3	4.8
tri-O-Ac-α-Ct	4.70	3.3×10^3	4.8
deacetylat prod of tri-O-Ac-α-Ct	4.70	3.6×10^3	4.9
di-O-Ac-α-Ct	4.70	1.0×10^4	5.5
deacetylat prod of di-O-Ac-α-Ct	4.70	1.0×10^4	5.5
α-Ct-formyl-L-Phe complex	4.12	2.6×10^3	4.7
Met-192-sulfoxide-α-Ct	4.12	6.4×10^3	5.2
α-Ct in 2% dioxane	4.12	5.7×10^3	5.1

[a] 25 °C, 0.01 M acetate buffer, 0.178 M NaCl.

schlieren and interference optics, electronic speed control, and RTIC temperature control unit. An aluminum-filled epon double-sector centerpiece with sapphire windows was used. The light of an AH-6 mercury lamp was filtered with a Kodak wratten 77A filter and a polarizing filter, and the interference patterns were recorded on Kodak IIG spectroscopic plates. A shutter was employed to allow a white light exposure of the air reference fringes to facilitate alignment of the plate on the microcomparator. Baseline patterns were determined after stopping the centrifuge, removing the rotor, and rocking it to destroy the concentration gradient in the cell, and then returning the rotor to operating speed and taking the baseline picture (Richards et al., 1968).

The vertical displacements of the average of three light fringes with radial distance were determined on a Nikon 6C microcomparator equipped with a digital micrometer and readout.[3] These values were punched onto a paper tape and read into a Wang 370 programmable electronic calculator. The point-average number and weight-average molecular weights and the value of the dimerization equilibrium constant at each radial position were calculated using a program developed by Aune (Aune and Timasheff, 1971). The partial specific volume was taken as 0.736 at 25 °C (Schwert and Kaufman, 1951; Lee and Timasheff, 1974). Fringe displacement was converted to concentration by $c = kf$, where c = concentration of protein, f = fringe displacement in micrometers, and $k = 0.000882$ g $l^{-1}\mu m^{-1}$ (Aune and Timasheff, 1971).

Circular dichroism experiments were carried out on a Cary Model 60 spectropolarimeter with a 6001 CD attachment, the slit width being programmed for a band resolution of 1.5 nm. Mean residue ellipticities, $[\theta]$, reported in deg-cm^2/dmol of residue, were calculated using a mean residue weight of 105. Three cells, with path lengths of 1.0, 0.1, and 0.01 cm, were used in different wavelength regions.

[3] Designed and constructed by Mr. Barkev Bablouzian of this Department.

Partial Specific Volume Measurements. The densities of the solvents and the protein solutions were measured with a Precision density meter DMA-02 (Anton Paar, Graz) following previously described procedures (Lee and Timasheff, 1974). The apparent partial specific volume, ϕ_2', at identical chemical potentials of solvent components in the protein solution and reference solvent, was then obtained as the average of values measured at several protein concentrations, since ϕ_2' was concentration independent.

Results

The following derivatives of chymotrypsin were prepared: methylhistidine-57-, destyrosine-146-, diacetyltyrosine-, and triacetyltyrosine-α-chymotrypsin, the deacetylation products of di- and triacetyltyrosine-α-chymotrypsin, Met-192-sulfoxide-, TPCK-, PMS-, and tosyl-α-chymotrypsin, α-chymotrypsin semicarbazide, α-chymotrypsinamidomethanesulfonic acid, and the slow and rapid activation products of chymotrypsinogen semicarbazide. Prior to use in the physical studies, the structural integrity of the various enzyme derivatives was checked by measuring their enzymatic activities and determining their circular dichroism spectra. All of the derivatives involving modification of tyrosine-146 were close to fully active. DIP-, TPCK-, tosyl-, and PMS-α-Ct were fully inactive, as was essentially the *N*-methylhistidine compound, the rate of which is known to be reduced by a factor of 10^3 to 10^5 (Nakagawa and Bender, 1970). The carboxyl-blocked enzyme had an activity of 30 to 40% relative to α-chymotrypsin, in accordance with the literature (Fersht and Sperling, 1973; Carraway et al., 1969; Abita et al., 1969; Johnson et al., 1976). Met-192-sulfoxide-α-Ct was 40% active in agreement with Taylor et al. (1973). The circular dichroism spectra of all derivatives were typical of native chymotrypsin both in the near- and far-ultraviolet regions.

The results of the association studies are summarized in Table I. It is evident that chemical modification of different residues of α-chymotrypsin affects dimerization differently. Let us examine them in turn.

Histidine-57. Methylation of histidine-57 at the $N^{\epsilon 2}$ position abolishes, within the limits of molecular weight measurements, the ability of α-chymotrypsin to dimerize in solution, even though this derivative exists in the dimer form in crystals (Wright et al., 1972). Sedimentation equilibrium in 0.178 M NaCl, 0.01 M acetate (pH 4.12) yielded an essentially monodisperse molecular weight distribution. In a typical experiment, the weight-average molecular weight averaged over the entire cell was 25 783, which is very close to the molecular weight of 25 245 calculated from the amino acid sequence (Brown and Hartley, 1966; Kaufman and Hartley, 1966). The measured association constant, 3×10^2 L/mol, is 100 times lower than that of the native α-chymotrypsin, which is 2.6×10^4 L/mol (Aune and Timasheff, 1970) and is not significantly different from zero. A similar result, shown in Figure 1, was obtained when the modifying agent was TPCK. These observations are in agreement with and quantitate those of Neet et al. (1974) (also Neet and Brydon, 1970), that both methylation of histidine-57 and its modification with TPCK greatly reduce the increase of the sedimentation coefficient with protein concentration of the modified enzyme relative to virgin α-chymotrypsin.

Tyrosine-146. Removal of tyrosine-146, which is the C-terminal residue of chain B, by treatment with carboxypeptidase A, resulted in a molecular weight distribution similar to that obtained with MeHis-57-α-Ct. In a typical experiment, the weight-average molecular weight averaged over the entire cell was 24 976, in very good agreement with the value of

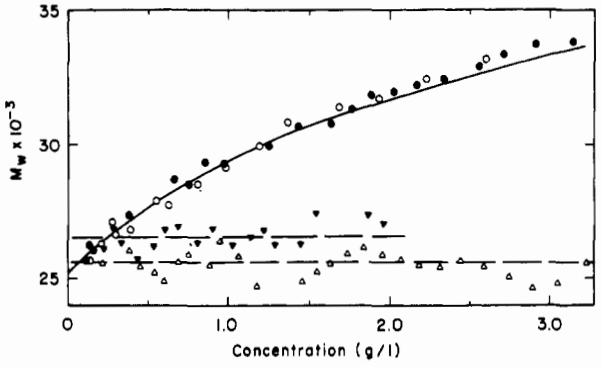

FIGURE 1: Dependence of the weight average molecular weight of α-chymotrypsin derivatives on protein concentration, at 25 °C in 0.178 M NaCl, 0.01 M acetate buffer (24 000 rpm; loading protein concentration 0.76 g/L): (●) tri-O-acetylated α-chymotrypsin, pH 4.70; (○) deacetylation product of tri-O-acetylated α-chymotrypsin, pH 4.70; (▼) slow activation product of the semicarbazide derivative of chymotrypsinogen, pH 4.12; (△) TPCK derivative of α-chymotrypsin, pH 4.12. The solid line is calculated for a dimerization constant of 2.9×10^3 L/mol; the dashed lines correspond to monomer molecular weights.

25 083 calculated from the amino acid sequence (Brown and Hartley, 1966; Kaufman and Hartley, 1966) corrected for the loss of one residue of tyrosine; and the dimerization constant was not significantly different from zero.

While this result fully quantitates the earlier observation of Gladner and Neurath (1954), it seemed of interest to examine the individual contributions to the dimerization reaction of the phenolic hydroxyl and α-carboxyl groups of this residue. For this purpose, α-chymotrypsin derivatives in which tyrosine-146 was O-acetylated or in which its carboxyl group was blocked were prepared and examined by sedimentation equilibrium.

Results of acetylation of α-chymotrypsin and some derivatives are compiled in Table II. As can be seen, the native enzyme can be acetylated to the extent of three tyrosines. Removal of tyrosine-146 reduces the maximum acetylation to two groups, indicating that Tyr-146 is not the residue totally inaccessible to this reaction. Interestingly, methylation of histidine-57 also reduces the degree of acetylation to two tyrosines, suggesting that this modification interferes with the acetylation of Tyr-146. This conclusion is supported by the finding that carboxypeptidase removal of Tyr-146 from MeHis-57-α-Ct does not decrease further the maximal degree of tyrosine acetylation. It would seem, therefore, that of the three tyrosines available to acetylation with *N*-acetylimidazole, Tyr-146 is the least accessible and is the last one to become modified.

In order to determine unequivocally the order in which the tyrosines are acetylated, tyrosine-146 was removed from both the di- and tri-O-acetylated derivatives of α-chymotrypsin, and the number of acetylated tyrosines remaining was determined as described above. Both destyrosine products were found to contain two O-acetylated tyrosines, leading to the conclusion that in the diacetylated derivative the Tyr-146 phenolic hydroxyl is free, since its removal does not change the degree of acetylation while it is blocked in the triacetylated protein. The total number of tyrosines remaining in each carboxypeptidase-treated derivative was checked spectrophotometrically by titration to pH 13.5, with the finding that treatment with carboxypeptidase had reduced the total number of tyrosines to three in both O-acetylated derivatives. Therefore, as suggested above, of the reactive tyrosines, the hydroxyl groups of tyrosine-94 and -171 are most readily accessible to acetylation at pH 7.5, while that of tyrosine-146 is less reactive. It is interesting to note that, in titration experiments, Tyr-146 is the second group to ionize,[4] while its ring carbons are most accessible to iodination (Dube et al., 1964, 1966). The extent of acetylation of aliphatic hydroxyls was determined by the difference in deacetylation at pH 7 (tyrosines only) and 11.5 (all labile acetyl residues). The results, summarized in the last column of Table II, indicate that the acetylation of serine and threonine residues was quite low in these derivatives.

TABLE II: Acetylation of α-Chymotrypsin Derivatives.

derivatives	*N*-acetylimidazole to enz molar ratio	Tyr acetylated	Tyr ionized at pH 13.5	aliphatic OH acetylated
α-ct	50–600	2	4	2
	900–1260	3	4	4
desTyr-146-Ct	130–1240	2	3	0.5
MeHis-57-Ct	120–700	2	4	2
desTyr-146-MeHis-57-α-Ct	120–400	2	3	2

The ability to dimerize of the di- and tri-O-acetyltyrosine derivatives of α-chymotrypsin was examined by sedimentation equilibrium in 0.178 M NaCl, 0.01 M acetate buffer at pH 4.70, to avoid hydrolysis of the acetyl groups at lower pH. It was found that the O-acetylation of two tyrosine residues had little effect on the dimerization of α-chymotrypsin, the standard free energy of the reaction, $\Delta G^\circ = -5.5$ kcal/mol, remaining essentially unchanged from that of the native enzyme. Acetylation of a third tyrosine, however, caused a profound decrease in the strength of the self-association. As seen from Table I, ΔG° of dimer formation of tri-*O*-acetyl-α-chymotrypsin is 0.9 kcal/mol less negative than that of the native enzyme and about equal to that of γ-chymotrypsin dimerization (Miller et al., 1971). Since, as shown above, the phenolic hydroxyl of Tyr-146 is free in the diacetylated derivative and is blocked only when the third acetyl is added on, these results mean that it is the acetylation of Tyr-146 which reduces the ability of α-chymotrypsin to dimerize.

In order to check whether the weakening of dimerization of α-chymotrypsin by O-acetylation of Tyr-146 is due specifically to the blocking of the hydroxyl group, rendering it incapable of interacting by hydrogen bond formation, or to a conformational change which occurs during acetylation, this material was deacetylated by treatment with hydroxylamine at pH 7. Ultracentrifugal examination of the obtained product resulted in a molecular weight distribution as a function of protein concentration identical with that of tri-*O*-acetyl-α-chymotrypsin, as shown in Figure 1. Therefore, deacetylation of the tri-O-acetylated derivative did not restore to the enzyme its original ability to dimerize. In fact, it is striking that the standard free energy of dimerization of both the tri-O-acetylated α-chymotrypsin and the product of its deacetylation, −4.8 kcal/mol, is the same as that of γ-chymotrypsin. On the other hand, di-O-acetylated α-chymotrypsin and its deacetylation product retain the ability to dimerize of the virgin enzyme. This seems to exclude exposure to the acetylation and deacetylation reaction conditions, or acetylation of groups other than tyrosine, as the cause of the decrease of the free energy of dimerization. It is much more likely that acetylation of the Tyr-146 hydroxyl causes a change in its environment which persists after deacetylation.

The participation of the α-carboxyl residues of Tyr-146 and

[4] M. J. Gorbunoff and S. N. Timasheff, to be published.

Asp-64 in the dimerization reaction was examined on α-chymotrypsin derivatives in which the carboxyls had been blocked either by semicarbazide or aminomethanesulfonic acid. To establish that neither the active-site serine nor tyrosine hydroxyl groups were modified during the carboxyl blocking, the derivative was treated with NH_2OH (Banks et al., 1969; Carraway and Koshland, 1968), which led neither to any increase in activity after 18 h nor to any change in the UV-absorption spectrum associated with the formation of a free tyrosine hydroxyl group. Fersht and Sperling (1973) had shown that modification of α-chymotrypsin with semicarbazide in the presence of proflavine results in a product in which all carboxyls, except for Asp-102, -194, and -64, are blocked. The first two are involved in the charge relay and the active structure-stabilizing internal ion pair, respectively, and are, therefore, inaccessible to chemical reaction. The lack of reactivity of Asp-64 was attributed by Fersht and Sperling (1973) to its involvement in an intermolecular salt bridge, in an assumed dimer. The α-carboxyl of tyrosine-146 is blocked in the product of this reaction. This was further verified in the present study by carboxypeptidase A treatment of this derivative, which released no free tyrosine, while it is known that a free carboxylic group is required for carboxypeptidase A cleavage (Smith, 1951). Since modification with semicarbazide results in a product with a loss in negative charge, a second derivative in which the charge is not affected was prepared using aminomethanesulfonic acid. In this derivative, each COOH is replaced by $CONH_2CH_2SO_3H$.

Sedimentation equilibrium examination showed (see Table I) that neither of these derivatives of α-chymotrypsin is able to dimerize, the weight-average molecular weight remaining close to that of the monomer across the entire ultracentrifuge cell. Therefore, blocking of the α-carboxyl of Tyr-146, while the carboxyl of Asp-64 is free, has an effect even more drastic on the acid dimerization of α-chymotrypsin than blocking of the Tyr-146 phenolic hydroxyl. It is also evident that the presence of a free carboxyl on Asp-64 is not sufficient to maintain the enzyme in the state of the dimer. At this point it seems pertinent to ask whether in α-chymotrypsin the failure of Asp-64 to react in the modification reaction is due to its shielding by dimer formation or to some other factors. Fersht and Sperling (1973) assumed that α-chymotrypsin exists as a stable dimer under the conditions of the carboxyl-modification reaction. Three strong arguments can be advanced to favor the opposite. First, had the enzyme remained as a stable dimer during the reaction, it is difficult to understand why it would not show any tendency to dimerize in the sedimentation experiment, which was preceded only by the removal of excess reagent by dialysis. Second, comparison of the conditions of the blocking reaction with the known pH and salt concentration dependence of the dimerization equilibrium constant (Aune and Timasheff, 1971; Aune et al., 1971), indicates that, under the conditions used by Fersht and Sperling (1973), 50–60% by mass of α-chymotrypsin remained in the state of monomer as a time average during the course of the reaction. The dimerization reaction being rapidly reequilibrated, all of the enzyme molecules should have been exposed to the reagent in monomer form many times during the 10-h long carboxyl-blocking process. As a result, the dimerization equilibrium per se should not be capable of protecting any residue from multiple contacts with the reaction medium. The third argument stems from the fact that the carboxyl-modification reaction was carried out in the presence of proflavin. Proflavin is a competitive inhibitor which binds in the active-site region of the enzyme (Bernhard et al., 1966) and has been shown to ligand only to monomeric α-chymotrypsin (Faller and LaFond, 1971; Gilleland and Bender, 1976). As a necessary consequence, proflavin must lower the dimerization constant of the α-chymotrypsin (Wyman, 1964; Lee and Timasheff, 1977). Thus, while the observation that Asp-64 is inaccessible to this chemical modification reaction in the active enzyme, while it is fully accessible in chymotrypsinogen, is very intriguing, the reason for this must be other than protection by dimerization. A possible explanation might be shielding of Asp-64 by bound proflavin during the modification of α-chymotrypsin, but its availability to the reagent in chymotrypsinogen which was reacted in the absence of proflavin. Furthermore, were Asp-64 indeed shielded from the solvent by being located in the surface of contact between the two molecules, a similar situation should have been true of Tyr-146, the α-carboxyl of which is universally agreed to interact in some manner with His-57 in the dimer.

In an attempt to prepare the reciprocal α-chymotrypsin derivative, in which the Asp-64 carboxyl is blocked and that of Tyr-146 is free, chymotrypsinogen was reacted with semicarbazide in the absence of proflavin, using the procedure of Fersht and Sperling (1973) who have shown that the carboxyl of Asp-64 is blocked in the product, with only the Asp-102 and -194 carboxyls remaining free. Chromatography of the carboxyl-modified chymotrypsinogen on CM-cellulose at pH 6.0 (Rovery, 1967) gave one peak which contained 70% of the starting protein. The modified zymogen was activated with trypsin under conditions of both slow and fast (Wilcox, 1970; Fersht and Sperling, 1973) activations, which lead to α- and δ- (or γ-) chymotrypsin, respectively, when virgin zymogen is used. Purification of the slow activation product on CM-cellulose gave two components, one eluting at 0.04 M citrate (55%) and the other at 0.06 M citrate (20%). On hydroxylapatite in pH 6.8 phosphate, only one peak was obtained which accounted for 57% of the loaded protein. When assayed with BTEE, all three fractions showed enzymatic activity which was 30% of purified commercial α-chymotrypsin, in agreement with the literature (Fersht and Sperling, 1973; Carraway et al., 1969; Abita et al., 1969; Johnson et at., 1976). In sedimentation equilibrium experiments, however, none of these products were found to dimerize, as shown in Table I and Figure 1. The reason for this inability to self-associate was probed by carboxypeptidase treatment of the active derivative purified on hydroxylapatite. No tyrosine was released, indicating that this product does not contain a terminal Tyr-146, a fact which was confirmed by titration to pH 13.5 which showed the presence of four ionizable tyrosines. Considering that the activation of the carboxyl-modified chymotrypsinogen proceeds 200 times faster than that of virgin Ctgen (Carraway et al., 1969; Abita et al., 1969) and that the active enzyme produced from it is devoid of a terminal tyrosine, it can be argued that the slow activation of carboxyl-modified chymotrypsinogen gives only a δ-chymotrypsin type of enzyme, with the covalent bond between Tyr-146 and Thr-147 intact. Since δ-chymotrypsin is known not to dimerize, the results of the sedimentation experiments are not surprising.

Further evidence that the modified enzyme prepared in this manner is in a δ-chymotrypsin conformation was obtained from examination of its circular dichroism spectrum in the 225–235-nm region. This band, which most probably reflects transitions of tryptophan and tyrosine residues,[5] is extremely sensitive to changes in environment and conformation. The CD spectra of the various semicarbazide derivatives at pH 3.1 are shown in Figure 2. Small, but reproducible, differences can be

[5] M. J. Gorbunoff and S. N. Timasheff, unpublished results.

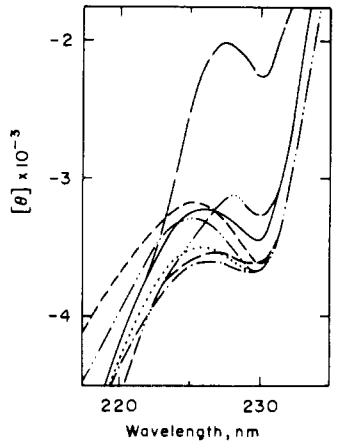

FIGURE 2: Circular dichroism spectra of chymotrypsin derivatives in 0.001 M HCl: (—) α- and γ-chymotrypsin; (- • • -) δ-chymotrypsin; (- - -) α-chymotrypsin semicarbazide; (- • -) γ-chymotrypsin semicarbazide; (- -) δ-chymotrypsin semicarbazide; (···) slow activation product of chymotrypsinogen semicarbazide; (— —) chymotrypsinogen; (- - ···· - -), chymotrypsinogen-semicarbazide.

seen. The CD spectra of native α-, γ-, and δ-chymotrypsins are almost identical, with a negative maximum at 230 nm and a trough at 225–226 nm. Reaction of α-chymotrypsin with semicarbazide does not produce any major changes in the spectrum. In the case of the δ and γ enzymes, identical changes in the spectrum are brought about by this reaction. The difference in ellipticities between the 230-nm peak and the 226-nm minimum greatly decreases, and the band remains little more than a flat shoulder on the steep background. The spectrum of the slow activation product of carboxyl-blocked chymotrypsinogen is found to be essentially identical with those of the γ and δ enzyme derivatives and different from that of the α derivative. Therefore, the activation product of carboxyl-blocked chymotrypsinogen not only maintains intact the Tyr-146–Thr-147 peptide bond but also results in an active enzyme which belongs to the γ, δ class of conformers, rather than to the α class.

An observation which seems pertinent to this discussion has been made by Steitz et al. (1969) that binding of formyl-L-tryptophan or formyl-L-phenylalanine to α-chymotrypsin displaces by several angstroms the Tyr-146 carboxyl of the dyad-related molecule from its position in the free enzyme. Such a displacement should affect the free energy of dimerization, if this group is indeed important in maintaining the dimeric structure. A sedimentation equilibrium experiment was performed, therefore, at pH 4.12 in the presence of 4×10^{-2} M formyl-L-phenylalanine, a concentration at which 90% of the enzyme should be liganded (Davis and Hess, 1974; Shultz et al., 1977). As shown in Table I, dimerization was greatly weakened, which is consistent with the notion that the exact position of this group plays a determining role in the strength of the self-association, although a contribution from steric interference with intermolecular contacts cannot be excluded.

Serine-195. The effect of blocking the active-site serine hydroxyl was examined, since Vandlen and Tulinski (1973) have established that it is required for the stabilization of the crystallographic dimer by salt-bridge formation with a sulfate ion. Three derivatives were examined, tosyl-, PMS-, and DIP-α-Ct. As shown in Table I, all three modifications greatly reduce the ability of the enzyme to dimerize but do not abolish it. The standard free energy of the reaction is less negative by approximately 1.5 kcal/mol than in the virgin enzyme. These results are in agreement with the reports of Smith and Brown (1952) and Gladner and Neurath (1953) on the ability of DIP-α-Ct to dimerize at pH 3.9. A qualitative examination of their sedimentation velocity data in the light of the Gilbert (1955) theory leads to an estimate of association constants of similar magnitude to that reported here. These results are also consistent with the elegant study of Horbett and Teller (1973) who found that acetylation of the Ser-195 hydroxyl did not inhibit the dimerization of α-chymotrypsin but that acylation of Ser-195 with increasingly bulkier groups progressively weakened the dimerization, suggesting steric hindrance in the site of intermolecular contact.

Methionine-192. Another intermolecular contact identified crystallographically is that between the Met-192 residues of the two monomers in the dimer. Their contribution to the stabilization of the solution dimer was probed in two ways. First, these residues were oxidized to the sulfoxide using the procedure of Taylor et al. (1973). Sedimentation equilibrium examination of the product showed a marked reduction in ability to dimerize (see Table I). The second probe was derived from the report of Steitz et al. (1969) that introduction of 2% dioxane causes a rotation by 180° of the sulfur of Met-192. Sedimentation equilibrium experiments at pH 4.12 in the presence of 2% dioxane showed that liganding of dioxane to α-chymotrypsin reduces the association constant to an extent similar to that obtained by oxidation of Met-192 sulfur to the sulfoxide (Table I). From these observations, it may be concluded that the contact between the dyad-related methionines is also formed in solution and that perturbation of the bulk or exact geometry of folding of this residue introduces steric interference in the intermolecular site of contact with a concomitant weakening of the interaction. One must recall that the analysis of the dimeric structure by Matthews et al. (1968) and by Sigler et al. (1968) was carried out on dioxane-liganded α-chymotrypsin.

Sulfate Ion. A major source of stabilization of the crystallographic dimer of α-chymotrypsin has been shown by Vandlen and Tulinski (1973) to be provided by a pair of sulfate ions which form bridges between the two enzyme molecules. On the other hand, in solution, sulfate ions are known to decrease the measured equilibrium constant of dimerization (Aune et al., 1971). This decrease has been attributed by Aune et al. (1971) to a salt effect on the activity coefficients of the charged groups (Robinson and Stokes, 1959) involved in the maintenance of the dimer. Alternately, the same values of the apparent dimerization equilibrium constants in the presence of Na_2SO_4 could have been obtained artifactually, if the values of the partial specific volumes used in the calculations of the data obtained in Na_2SO_4 media, which were assumed by Aune et al. (1971) to be identical with those in the NaCl-containing solvent, had been too low, there being no change in the extent of dimerization. In such a case, it can be calculated that the value of the effective partial specific volume in 0.2 M Na_2SO_4 should be greater by ca. 0.025 mL/g than that in 0.2 M NaCl. Such a situation would arise if the degree of hydration of α-chymotrypsin were greater by approximately 1 g of water/g of protein in 0.2 M Na_2SO_4 than in 0.2 M NaCl (Aune et al., 1971). Although highly unlikely, this possibility was nevertheless tested by measuring the apparent partial specific volume of α-chymotrypsin, ϕ_2', at conditions at which the chemical potentials of solvent components are identical in the solvent and the protein solution (Cassassa and Eisenberg, 1964; Lee and Timasheff, 1974) in 0.2 M NaCl and 0.2 M Na_2SO_4 in 0.01 M pH 4.3 acetate buffer, following dialysis of the protein against the solvent. The results are summarized in Table III. The values of $\phi_2' = 0.739$ mL/g in 0.2 M NaCl and

TABLE III: Apparent Partial Specific Volume of α-Chymotrypsin in Various Salts at 25 °C.

solvent	ϕ_2' (mL/mg)
10^{-3} M HCl, 0.1 M KCl	0.735 ± 0.002
pH 4.3, 0.01 M acetate buffer	0.733 ± 0.002
same + 0.2 M Na_2SO_4	0.737 ± 0.002
same + 0.2 M NaCl	0.739 ± 0.003
same + 0.2 M $NaClO_4$	0.726 ± 0.003

0.737 in 0.2 M Na_2SO_4 are identical within experimental error. The increase in ϕ_2', by approximately 0.025 mL/g, which would have been required had the decrease in the measured dimerization constant been artifactual, is not observed. The decrease in the dimerization constant must, therefore, be regarded as real, supporting the conclusion of Aune et al. (1971) that, in dilute solution, sulfate ions weaken the intermolecular bond by an electrostatic thermodynamic effect. On the other hand, in 0.15 M $NaClO_4$, there appears to be a real decrease in ϕ_2', which suggests a decrease in the preferential hydration of the enzyme, and is consistent with stabilization of charge–charge interactions, as proposed by Aune et al. (1971).

Discussion

The experiments described in this paper show clearly that the dimerization of α-chymotrypsin can be readily perturbed by the chemical modification of a number of groups implicated in the mechanism. Thus, methylation of the $N^{\epsilon 2}$ nitrogen of His-57 totally suppresses dimerization, as does blocking of the α-carboxyl of Tyr-146, even though the carboxyl of Asp-64 remains free. Acetylation of the Tyr-146 hydroxyl reduces the free energy of the association to a value identical with that of γ-chymotrypsin. Deacetylation does not reverse the situation. On the other hand, acetylation of the tyrosine hydroxyls of two residues not located in the surface of intermolecular contact has no effect on this reaction nor does their deacetylation. It appears, therefore, that the observed effects of chemical modification on the acid dimerization in solution of α-chymotrypsin are specific.

While the effect of blocking the α-carboxyl of Tyr-146 strongly supports the earlier conclusion that this group is involved in the mechanism of dimer formation, the possibility must be considered that this is simply the result of an increase in the nonspecific electrostatic repulsion between the protein subunits due to the increased net positive charge on the protein, even though the nonspecific electrostatic free energy was found to be small for the virgin enzyme (Timasheff, 1969; Aune and Timasheff, 1971). This was calculated, with the assumption that all but three carboxyl residues are blocked. At pH 4.12 the α-chymotrypsin semicarbazide derivative should have a net charge of +20 (Marini and Wunsch, 1963). Application of the Verwey–Overbeek (1948) equation (Timasheff, 1966, 1969, 1970) results in a nonspecific electrostatic repulsion contribution of ~0.5 kcal/mol. This would reduce the dimerization association constant to $\sim 1.0 \times 10^4$ L/mol at pH 4.12 or well within the range measurable by our methods. This conclusion was verified by replacing the semicarbazide in the carboxyl-blocking reaction with aminomethanesulfonic acid. The product of this reaction, which has a net charge identical with that of the virgin enzyme, is also fully devoid of the ability to form the acid dimer, supporting the conclusion that the inability of carboxyl-blocked α-chymotrypsin to dimerize is the specific consequence of the blocking of Tyr-146. A change in conformation induced by the blocking of carboxyls of α-chymotrypsin can also be excluded, since both derivatives have the expected enzymatic activities, while their CD spectra are identical with that of the native enzyme.

Results of the study with the O-acetylation products might suggest that a free hydroxyl on Tyr-146 is required in this reaction, since its blocking results in a decrease of the free energy of dimerization by 20%, i.e., 0.9 kcal/mol. Indeed, in the crystal dimer, in 75% saturated ammonium sulfate, this group is known to be involved in the intersubunit interaction via a hydrogen bond to the bridging sulfate ion (Vandlen and Tulinski, 1973). It seems, however, that in dilute solution an alternate explanation should be sought. First, it is most improbable that a similar bridge via an anion exists in solution, since the present sedimentation results were obtained in a dilute NaCl medium in the absence of divalent anions. Furthermore, as shown by Aune et al. (1971), in dilute solution, substitution of sulfate ions for chloride in the medium weakens the dimerization, an effect which can be accounted for by the effect of sulfate ions on the activity coefficients of charged groups (Robinson and Stokes, 1959). The alternate possibility, namely, that of an artifact due to failure to take into account in the data analysis an increase in protein hydration by 1 g of water/g of α-chymotrypsin when the medium is changed from 0.2 M NaCl to 0.2 M Na_2SO_4, without any change in the extent of dimerization, has been excluded by the partial specific volume measurements described above. Furthermore, measurements of the binding of anions to α-chymotrypsin as a function of pH (Friedberg and Bose, 1969) did not reveal any abrupt increase in the binding of either sulfate or chloride ions in the pH region of dimerization. Such an increase would be expected, if strong binding of the anions, such as would result from the formation of a sulfate bridge, were to occur during dimerization (Wyman 1964; Lee and Timasheff, 1977). A further argument against the existence in solution of the crystallographic sulfate bridge between the α-chymotrypsin molecules is provided by the observations that blocking of the Ser-195 hydroxyl by formation of the tosyl, PMS, or DIP derivatives does not eliminate dimerization, as well as by the reports of Neet and Brydon (1970) and of Horbett and Teller (1973) that acetylation of the Ser-195 hydroxyl does not interfere with dimerization.

An intriguing interpretation of the diminution of the association constant when the hydroxyl of Tyr-146 is acetylated can be drawn from the unexpected results obtained with the products of the deacetylation reaction. It is quite striking that O-acetylation of Tyr-146 not only does not abolish the dimerization reaction of α-chymotrypsin but, in fact, it reduces the association constant to a value equal to that of γ-chymotrypsin, a value which remains unchanged after deacetylation. This highly reproducible result is specific for the O-acetylation of Tyr-146, since O-acetylation to the extent of two tyrosines does not affect significantly the free energy of dimerization, nor does the deacetylation reaction as such. α-Chymotrypsin and γ-chymotrypsin are stable conformers of the same chemical species; the only known difference in their solution properties resides in the strength with which they dimerize in the pH 4 region. X-ray crystallographic analysis has shown that the principal structural difference between them is in the position of Tyr-146 (Freer et al., 1970), as have iodination experiments (Sigler et al., 1968; Wright and Weber, 1972). Matthews et al. (1968) have found that the α carbon of Tyr-146 is slightly displaced in γ-chymotrypsin from the position it occupies in the α enzyme. Indeed, Wright et al. (1968) have proposed that the difference between α- and γ-chymotrypsins resides in a difference in the conformations of the carboxyl termini of the B chain (Tyr-146) in the two molecules. It is interesting to speculate that acetylation of the Tyr-146 hy-

droxyl of α-chymotrypsin induces this residue to shift its position to that of the γ conformer, this form of the enzyme persisting after deacetylation. This hypothesis is fully consistent with the finding of Wright and Weber (1972) that the $\alpha \rightleftharpoons \gamma$ interconversion is possible only when Tyr-146 is protonated. This effect should be even more pronounced on O-acetylation. The Tyr-146 hydroxyl, therefore, need not necessarily be integrally involved in the mechanism of solution dimerization. The difference in the abilities of α- and γ-chymotrypsins to dimerize could then reside strictly in the difference in location of the Tyr-146 carboxyl. The plausibility of this position was tested by an approximate calculation of the displacement of this residue necessary to account for the difference in the standard free energies of dimerization of the two enzymes. At pH 4.12, $\Delta G°$ decreases from -6.0 kcal/mol for α-chymotrypsin to -5.0 kcal/mol for the γ form of the enzyme. If the electrostatic free energy of attraction, ΔG^e, between two charges in the intersubunit area of contact is approximated by an unscreened Coulombic potential, then the difference between the values for two conformers which differ only by the distance between two pairs of interacting charges is given by:

$$\delta(\Delta G^e) = \Delta G_\alpha{}^e - \Delta G_\gamma{}^e = \frac{-2e^2}{D}\left(\frac{1}{R_1} - \frac{1}{R_2}\right) \qquad (1)$$

where e is the electronic charge, D is the dielectric constant in the intersubunit zone of contact, and R_1 and R_2 are the distances between the two interacting charges in the two forms of the enzyme. Setting $D = 20$ in the area of subunit contact (Kirkwood and Westheimer, 1938; Tanford, 1957; Timasheff, 1966, 1970), $\delta(\Delta G^e) = -1.0$ kcal/mol, and $R_1 = 3.5 \times 10^{-8}$ cm (Birktoft et al., 1972), the difference in crystallographic distance between Tyr-146 and His-57 in the two conformations of the chymotrypsin dimer ($R_1 - R_2$) is calculated to be only 0.4 Å; i.e., the distance between the two charged groups in γ-chymotrypsin is only 0.5 Å greater than in the α conformer. Using an unrealistically high dielectric constant value of 80 results in ($R_1 - R_2$) = 2.4 Å. An identical result is obtained if one introduces full Debye-Hückel screening into eq 1. Even though these calculations are highly approximate, they do lead to the conclusion that a difference between the space coordinates of the α-carboxyl of Tyr-146 of 1–3 Å between α- and γ-chymotrypsins, which is fully consistent with the crystallographic results, is sufficient to account for the differences in their abilities to dimerize.

At this point, it seems of interest to consider the origins of the different conformational states of Tyr-146 in α- and γ-chymotrypsin. These can be traced to their pathways of formation (Wright et al., 1968). γ-Chymotrypsin is obtained by the excision of the Thr-147–Asn-148 dipeptide from δ-chymotrypsin. Its conformation of Tyr-146 is essentially identical with that of δ-chymotrypsin (Wright et al., 1968; Matthews et al., 1968; Cohen et al., 1969), since the difference Fourier maps between these two forms show only a single peak at the locus of the missing dipeptide. It is therefore determined by the conformational changes which occur when chymotrypsinogen is cleaved between Arg-15 and Ile-16 with the B and C chains still covalently joined to each other. In the δ-chymotrypsin conformation, the region from Tyr-146 to Ala-149 is evidently not strained, since cleavage of the chain does not result in any spacial rearrangment. α-Chymotrypsin is formed by first cleavage of the Tyr-146–Thr-147 bond of chymotrypsinogen, followed by hydrolysis of the Arg-15–Ile-16 bond. Its terminal tyrosine conformation is determined to a great extent, therefore, when the A and B chains are still in the chymotrypsinogen conformation. Further cleavage of the Arg-15–Ile-16 bond either does not affect the Tyr-146 conformation or, if it does, it does not bring it to the δ, γ state. Evidently, in this pathway, cleavage of the chain in the Tyr-146–Ala-149 region relieves some strain, with the resulting displacement of Tyr-146. The (δ, γ) and α conformations of Tyr-146 cannot be greatly different, although the phenolic hydroxyl is less exposed in the α than the γ conformer, being less readily titrated (pK = 10.5 in γ and 11.3 in α) and less accessible to the cyanuration reaction (Gorbunoff, 1971). Nevertheless, the energy barrier separating them must be considerable, since they are not readily interconvertible.

The results on the acid dimerization of α-chymotrypsin in solution, reported in this paper, are still fully consistent with the proposal of Aune and Timasheff (1971), supported by Horbett and Teller (1974), that a prime interaction in α-chymotrypsin dimerization is ion pair formation between the imidazolium ion of His-57 and the terminal carboxylate of Tyr-146. Vandlen and Tulinski (1973), while recognizing this interaction as consistent with the crystallographic structure, propose an alternate interaction by hydrogen bonding between the un-ionized form of the Tyr-146 carboxyl and the carbonyl oxygen of His-57. They have suggested that this mode of interaction is consistent with the burial of the carboxylic acid groups of Tyr-146 in the dimeric structure which they state should have a pK higher than that ordinarily expected for a terminal carboxyl. Indeed, following their observation that in α-chymotrypsin crystals the dimer contacts, including the sulfate ion bridge, persist up to pH 6.0, these authors (Vandlen and Tulinski, 1973) suggest that the terminal carboxyl of Tyr-146 may become ionized only above pH 6.0. This interpretation, while possibly valid for the crystal state, presents a number of difficulties for the state in solution. First, it is true that a carboxyl buried in the state suggested by Vandlen and Tulinski (1973) should have a raised apparent pK. However, if the burial involves the formation of an ion pair, the effect should be the opposite; namely, the pK of the carboxyl should be depressed. Our earlier thermodynamic analysis of this system (Aune and Timasheff, 1971) showed that dimerization was accompanied by a shift in pK of a group from 3.6 in the monomer to 2.4 in the dimer. These values are those expected for a terminal carboxyl interacting via ion pair formation. Second, we have shown that methylation of the His-57 ring renders dimerization in solution impossible. In the crystal, however, the dimeric structure is preserved essentially intact (Wright et al., 1972), His-57 is displaced by only 0.3 Å and it is in close proximity to the C-terminal carboxylate of Tyr-146 of the dyad-related molecule. A hydrogen bond between this carboxylate and the carbonyl oxygen of His-57 should be formed just as easily in MeHis-57-α-Ct as in the virgin enzyme. The inability of this enzyme to dimerize in solution leads to the conclusion that it is the removal of the site of protonation from His-57, and not steric effects induced by the introduction of a methyl group into the region of contact, which interferes with the dimerization. Indeed, the steric hindrance which results from the introduction of much bulkier groups into the region of intermolecular contact, by formation of the tosyl, PMS, or DIP derivatives of α-chymotrypsin, does not abolish the ability of the enzyme to dimerize but only reduces the dimerization standard free energy by ~1.5 kcal/mol (see Table I). Introduction into the same region of an acetyl group by acetylation of Ser-195 does not affect the solution reaction at all (Horbett and Teller, 1973). Third, Vandlen and Tulinski (1973) report that the crystallographic contacts about dyad axis A persist to the pH 6–7 region, the sulfate ion bridge remaining intact, but the His-57–Tyr-146 interaction shifting from the H-bonded mode to the ionic mode which we had proposed for the

acid pH dimerization. Such a situation cannot exist in solution. It is known that in the higher pH region, α-chymotrypsin undergoes an association reaction in solution (Rao and Kegeles, 1958; Massey et al., 1955). Its mode, however, must be different from that in the acid-pH region, as shown by Neet et al. (1970, 1973). At pH 6.2, δ-chymotrypsin, which does not dimerize at pH 4.0, associates as readily as α-chymotrypsin. Furthermore, a number of derivatives of α-chymotrypsin modified on Ser-195, Met-192, and His-57, which do not dimerize at pH 4.0, associate as extensively as the native enzyme at pH 6.2. It seems particularly pertinent to the present discussion that methylation of His-57 of α-chymotrypsin does not interfere with the pH 6.2 self-association. This precludes its involvement in the intersubunit contact by ion-pair formation with the Tyr-146 carboxylate at pH 6.2, as proposed by Vandlen and Tulinski (1973) from the crystal structure in 75% saturated ammonium sulfate.

Crystal–Solution Structural Relationships. The above discussion leads directly to the question: Are the structures of α-chymotrypsin in solution and in crystals identical? While there is no doubt that the overall three-dimensional structures must be the same in their general features, the present discussion leads to the conclusion that there are serious differences in the detailed positions and local interactions of surface residues. These differences might not involve large displacements of groups, but nevertheless they can be highly significant for molecular function, such as is exhibited by self-association. For example, in the crystal, most derivatives of α-chymotrypsin, such as MeHis-57-, tosyl-, PMS-, or DIP-α-Ct, the dioxane complex, and the complex with formyl-L-phenylalanine, appear to form dimers with equal readiness (Wright et al., 1972; Sigler et al., 1966; Steitz et al., 1969). In solution, however, the same derivatives exhibit varying degrees of difficulty in forming dimers, if not total inability to dimerize. From their very careful examination of the α-chymotrypsin asymmetric crystallographic dimer, Tulinski and co-workers (Vandlen and Tulinski, 1973; Tulinski et al., 1973; Mauridis et al., 1974) have presented a most interesting analysis of the interactions which take place in crystals in 75% saturated ammonium sulfate and of the changes in these interactions with change in pH. Extrapolation to dilute solution, in the absence of sulfate, is, however, replete with difficulties. First, the contacts which exist in crystals at pH 6.0 cannot be the same as those formed in solution aggregates at the same pH.[6] In the acid-pH region, the differences are much more subtle. The interacting groups are essentially the same, but the details of the contacts which they form are not. In both states, His-57 of one molecule interacts with the Tyr-146 carboxyl of the other. The conclusion drawn from the crystallographic study favors hydrogen-bond formation between a protonated carboxyl of Tyr-146 and the carbonyl oxygen of His-57, although direct contact between the His-57 ring and the ionized Tyr-146 carboxyl is not excluded. The solution results can be readily explained in terms of the second mode of interaction but are inconsistent with the H-bond formation. Crystallographic results indicate the formation of an ion pair between the terminal amino of Ala-149 and the carboxylate of Asp-64. While such a pair can contribute to the overall free energy of the self-association in solution, it can hardly account for the pH dependence. A terminal amino residue ionizes with an intrinsic pK of 7.5 (Tanford, 1962). Therefore, were the pH dependence determining ion pair an α-carboxyl and an α-amino, as would be the case for the Asp-64–Ala-149 interaction, the alkaline side decrease in free energy in the pH profile of dimer formation should take place at a considerably higher pH than the observed region of pH 4.5–5. Rigorous thermodynamic analysis (Aune and Timasheff, 1971) has shown that the basic group involved in the ion pair has a pK of ionization in the monomeric state of ca. 5.0–5.2. This is compatible with a histidine residue or a carboxyl but not with a terminal amino. Finally, the crystal dimer owes much of its stability to a sulfate ion bridge between the phenolic hydroxyl of Tyr-146 of one molecule and the Ser-195 hydroxyl and Gly-193 imino of the other molecule. In solution, this interaction is totally excluded. It seems very reasonable that, in order to permit the phenolic hydroxyl to interact in the energetically most favored mode, interaction with the sulfate ion in the crystal dimer orients the ring of Tyr-146 somewhat differently from its position in solution. Such displacements of the tyrosine ring could easily be translated into small displacements of the carboxyl group of this residue. Indeed, Wright and Weber (1972) have concluded that the appearance of the sulfate binding interstitial site in their crystals is an artifact of the crystal environment and is unlikely to resemble the chemistry in solution. A reasonable position of Tyr-146 in the dilute solution dimer would be one which favored interaction with the imidazole ring of His-57, whose positive character would not be partly screened by the sulfate ion as it is in the crystals (Vandlen and Tulinski, 1973). The suggestion that the most favorable conformational free energy of the crystal dimer stabilized by two sulfate ions is obtained when the residues in the surface of contact are displaced somewhat from their positions in the solution dimer is well supported by the persistence in the crystal at pH 6.0 of the pH 4 contacts when these are stabilized by the two sulfate ions (Vandlen and Tulinski, 1973). In solution, such contacts do not exist at this pH. It seems quite probable, therefore, that the differences observed between the crystal and solution conformations of α-chymotrypsin are the result of an energy barrier imposed by the sulfate ions in the crystal.

These differences cannot be large and, were it not for the solution dimerization reaction, they would not have been noticed. Their significance, however, becomes more salient with the realization that the structural area of the enzyme involved in the dimerization is that of the active site. One must, therefore, exert extreme caution in extrapolating to the dilute solution state mechanistic conclusions drawn from the structure of the active site of α-chymotrypsin in the crystallographic asymmetric dimer. A better object of crystallographic studies should be δ-chymotrypsin in which the structure of the active-site region is not perturbed by intermolecular contacts due to self-association. That differences exist between the solution and crystal conformations of surface residues in proteins is not surprising; in fact, simple thermodynamic considerations suggest that this situation should be the rule rather than the exception. Indeed such a difference has been reported for carboxypeptidase A (Johansen and Vallee, 1975) and strongly implied in ribonuclease (Winstead and Wold, 1965).

Finally, in conclusion one must comment on a very important finding of Vandlen and Tulinski (1973) that the crystal dimer is not symmetrical about dyad axis A. Horbett and Teller (1973) have suggested that the low pH dimer might be an enzyme–product complex. The observed difference between the structures of the two halves of the crystallographic dimer makes this proposal most intriguing.

[6] It is not clear whether the crystals examined by Tulinski and co-workers at the higher pH values are in true thermodynamic equilibrium with their environment. These were obtained by soaking crystals, grown at lower pH, in higher pH buffers containing 75% saturated ammonium sulfate. Due to the slow kinetics of conformational interconversion in the crystal state, the resulting systems may, therefore, not have reached a state of thermodynamic equilibrium, but they may represent metastable intermediates which would have no counterpart in dilute solution.

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Dedication

This paper is dedicated to the memory of the late Professor Joseph F. Foster.

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Charge Shift Optical Probes of Membrane Potential. Theory†

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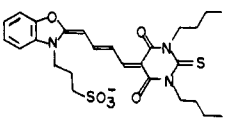
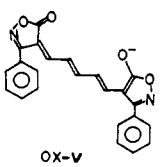
ABSTRACT: The chromophores of a series of known and unknown probes of membrane potential are subjected to molecular orbital calculations. These calculations are used to characterize the charge distribution and excitation-induced shift of electron density in the chromophores. This is used to predict or rationalize the magnitude of an electrochromic response to membrane potential. The predictions are consistent with more rigorous calculations on several selected systems as well as with the available experimental data. Emerging from the survey is a variation on previously considered forms of electrochromism involving a simple migration of the charge in an ionic chromophore. The intrinsic amphipathic structures of some of these systems may make them especially well suited for the construction of well oriented, highly responsive probes. A particularly promising charge-shift chromophore is the 4-(*p*-aminostyryl)pyridinium cation.

It has been well demonstrated (Tasaki et al., 1969a,b; Tasaki, 1974; Cohen et al., 1974; Ross et al., 1974, 1977; Conti, 1975) that electric potential changes, monitored by microelectrodes, are paralleled by changes in the absorption or fluorescence spectra of a wide variety of dyes applied to the squid giant axon. This pioneering work has encouraged many investigations into the utility of optical probes for the measurement of potential across the membranes of a variety of cells, organelles, and vesicle preparations that are inaccessible to microelectrodes (for recent reviews, see Chance et al., 1974; Chance, 1975; and Waggoner, 1976).

Unfortunately, dyes that appear particularly sensitive in one membrane system do not necessarily respond well in another. For example, M-540 (originally dye "I" in Cohen et al., 1974)



M-540



OX-V

is unresponsive in chromatophores (Chance and Baltscheffsky, 1975) but is quite sensitive in submitochondrial particles (Chance, 1975; Chance and Baltscheffsky, 1975). On the other hand, OX-V (Smith et al., 1976) is quite successful on chromatophores but inferior to M-540 on submitochondrial particles or squid axon (Chance, 1975; Chance and Baltscheffsky, 1975; OX-V is designated MC-V in these references). The reason for this problem is probably the diversity of mechanisms which are available to couple a spectral response to a change in membrane potential. Some of the mechanisms which have been postulated include:

(1) A potential-dependent partition of the dye between the cell interior and the external medium. Concentration of dye in the interior results in the formation of aggregates which are not fluorescent. Evidence for this mechanism has been found for the cationic highly permeant cyanine dyes in cell suspensions with a response time on the order of seconds (Sims et al., 1974).

(2) A potential-dependent partition between the external medium and the membrane. This requires an initial electrical potential to assure an asymmetric distribution of dye. This mechanism may have response times as fast as tens of microseconds, and the spectral change is thought to arise from the formation of dimers in the aqueous phase (Waggoner, 1976; Waggoner et al., 1977) [a solvatochromic effect may also be important (Pick and Avron, 1976)]. This mechanism has been associated with the cyanines in the squid axon (Cohen et al., 1974; Ross et al., 1974) by Waggoner (1976) and in chromatophores by Pick and Avron (1976).

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