Tyrosine Environment Differences in the Chymotrypsins*

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ABSTRACT: The accessibilities in solution of the tyrosine residues in δ-, γ-, and α-chymotrypsins have been compared. The δ and γ enzymes were found to have two tyrosine residues titrating with a pK_{app} of 10.5, one residue with pK_{app} values of 11.9 and 12.4 in δ and γ, respectively, and one deeply buried residue which is not identical in the two proteins. Probing by chemical modification with cyanuric fluoride showed that all three enzymes have three reactive residues, each of a different degree of reactivity. As a general pattern, the γ-chymotrypsin tyrosines are most reactive, while those in the α enzyme are least reactive.

The effect of cyanuration on the biological activity has been examined, the loss of activity increasing in the order $\delta < \alpha < \gamma$.

X-Ray crystallographic studies of the chymotrypsin family of enzymes and the zymogen have shown that the process of zymogen to enzyme activation is not accompanied by gross structural changes (Matthews *et al.*, 1967, 1968; Kraut *et al.*, 1967; Sigler *et al.*, 1968; Wright *et al.*, 1968; Freer *et al.*, 1970). The structural changes which occur in the course of activation have been associated with the primary activation process, since comparison of low resolution structures of π-, δ-, and γ-chymotrypsins with the 2-Å resolution structure of the α form (Freer *et al.*, 1970) has not revealed any significant differences in the folding of the polypeptide chains other than the excision of the Thr-147-Asn-148 dipeptide where applicable (Kraut *et al.*, 1967; Wright *et al.*, 1968; Matthews *et al.*, 1968; Cohen *et al.*, 1969; Davies *et al.*, 1969). Furthermore, there appear to be no significant differences in the overall size or shape of the molecules in solution (Krigbaum and Godwin, 1968). The activation process is accompanied, however, by a decrease in specific levorotation (Neurath *et al.*, 1956), which is probably related to the differences between the circular dichroism and optical rotatory dispersion spectra of the zymogen and the enzyme (Biltonen *et al.*, 1965; Raval and Schellman, 1965; Fasman *et al.*, 1966; Hess, 1969; McConn *et al.*, 1969; Timasheff, 1970a). Conformational differences between the zymogen and the enzyme, as well as between α-, δ-, and γ-chymotrypsins, have also been detected in nuclear magnetic resonance studies (Hollis *et al.*, 1968; Lumry and Biltonen, 1969). The crystallographic studies have demonstrated that the α carbon of Tyr-146 becomes displaced by 4.6 Å during the zymogen to α-enzyme transformation, with no indication that other tyrosines are similarly displaced. γ-Chymotrypsin, which is identical with the α form in amino acid sequence, seems to differ from the α form in the region of Tyr-146 (Sigler *et al.*, 1968; Wright *et al.*, 1968; Matthews *et al.*, 1968). The asymmetric crystallographic unit of the α enzyme contains two molecules related by a dyad axis of symmetry, while those of the zymogen, π, δ, and γ forms contain only one. Since the ability to dimerize in the crystal requires that Tyr-146 be terminal (Matthews *et al.*, 1967, 1968; Wright *et al.*, 1968; Sigler *et al.*, 1968; Cohen *et al.*, 1969), the crystallographic similarity of the γ form to the zymogen, π, and δ forms, in which Tyr-146 is part of the chain, is unexpected. The dimerization of α-chymotrypsin in the crystalline state involves a pair of identical interactions between Tyr-146 of one molecule and His-57 of the other molecule about dyad axis of symmetry A and results in the burial of Tyr-146. This is strikingly demonstrated by the difference between the iodination of the α form in the crystal and in solution (Matthews *et al.*, 1967, 1968; Sigler, 1970; Sigler *et al.*, 1968; Dube *et al.*, 1964, 1966). Since the iodination results for the γ form in the crystal (Cohen *et al.*, 1969) and the α form in solution are identical, the question arises whether the dissimilarity of the environment of Tyr-146 is maintained in solution. That this might be so is suggested by the ability of the chymotrypsins to dimerize at acid pH. α-Chymotrypsin dimerizes in the pH range between 3.5 and 5.5 (Schwert, 1949; Steiner, 1954; Gladner and Neurath, 1954; Egan *et al.*, 1957; Rao and Kegeles, 1958; Kézdy and Bender, 1965; Aune and Timasheff, 1971), while γ-chymotrypsin exhibits, at best, a much weaker dimerization (Schwert, 1949; Aune and Timasheff, 1971) *i.e.*, a behavior intermediate between that of the α form, on one hand and the zymogen and the δ enzyme, which do not dimerize, on the other hand (Dreyer *et al.*, 1955; Neet and Brydon, 1970; Horbett and Teller, 1970; Aune and Timasheff, 1971). While the X-ray studies suggest that the major effects of the zymogen to enzyme transformations upon the tyrosine residues are limited to Tyr-146 and that, except for a difference in this residue between the α and γ forms, the α carbons of the tyrosines are in essentially similar environments in the zymogen and the various enzyme forms, subtle differences between the phenolic hydroxyl groups would not be detected. It seemed, therefore, of interest to probe in solution the environments of these groups.

The environment of tyrosine residues in proteins can be probed in different ways (Timasheff and Gorbunoff, 1967; Timasheff, 1970a), such as titration, difference spectroscopy, and chemical modification. Cyanuric fluoride (CyF) (Kurihara *et al.*, 1963) appears to be a reagent of choice for the modification of tyrosines, since, among the tyrosine modifiers, it satisfies most rigorously the criterion of nonrandom action, probably as a result of the rigid geometric requirements of the transition state (Gorbunoff, 1970).

In the present study, δ-, γ-, and α-chymotrypsins were

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titrated spectrophotometrically in 1 M $KHCO_3$ buffer with and without 10% dioxane. The states of the tyrosine residues of these three enzymes were examined with CyF and compared to their titration properties and with corresponding data on the zymogen.

Materials and Methods

Materials. α-Chymotrypsin, three-times crystallized (lot CDI8LK), δ-chymotrypsin (lot CDD602), γ-chymotrypsin, twice crystallized (lot CDG6204-5), and Determatube *N*-Bz-L-TyrOEt were purchased from Worthington Biochemical Corp. The other reagents used were as described before (Gorbunoff, 1969).

Methods. Spectroscopic measurements were made at room temperature on a Cary Model 14 recording spectrophotometer. The pH's were measured at room temperature with a Radiometer 28 pH meter. Protein concentrations were determined spectroscopically in 0.001 M HCl containing 10% dioxane at 280 nm. The absorptivity value of 20.0 dl/g cm was used for α-chymotrypsin (Marini and Wunsch, 1963); for δ-chymotrypsin a value of 18.57 and for γ-chymotrypsin that of 20.1 dl/g cm were determined, using concentrations obtained from dry weight measurements of the proteins which had been dialyzed overnight *vs.* 0.001 M HCl. The molecular weights were taken as 25,245 for the α and γ enzymes and as 25,443 for δ, based on their amino acid compositions (Dayhoft and Eck, 1967–1968).

Spectrophotometric titrations were carried out by the difference spectral technique (Wetlaufer, 1962) in 1 M $KHCO_3$ buffer, with and without 10% dioxane. The ultraviolet spectra were recorded against protein solutions dissolved in 0.001 M HCl with and without 10% dioxane immediately after mixing and at 5-min periods thereafter. The optical densities within 15–20 min after mixing were taken as final values for all three chymotrypsins at pH 13.5. The near-ultraviolet maxima of the difference spectra were found to be at 298.0 nm for δ- and γ-chymotrypsins and at 299.0 nm for α-chymotrypsin. All three enzymes had a difference spectrum maximum at 245.0 nm. The number of ionized tyrosine residues was calculated using extinction coefficients for the phenoxide ion of 2300 at 295 nm and 10,900 at 245 nm (Crammer and Neuberger, 1943; Sage and Singer, 1962; Hermans, 1962).

Reaction with CyF. The procedure used was the same as described previously (Gorbunoff, 1967, 1968), except that the reaction time was limited to 45 min. The pH of the reaction mixture was then adjusted to 13.5. The ultraviolet spectrum was recorded in 2-cm cells between 290 and 340 nm after 2 min against a standard of the same concentration in 0.001 M HCl containing 10% dioxane, since it was found that, in the case of all three proteins, there was no difference in ultraviolet absorption in the 290–340-nm region between untreated protein dissolved in 0.001 M HCl containing 10% dioxane and the CyF-treated protein adjusted to pH 7.0. All three proteins were used at concentrations of 5 mg/ml.

The number of moles of tyrosine residues which had reacted under any given conditions of pH, CyF concentration, and temperature was calculated as before (Gorbunoff, 1969). The ultraviolet spectra of the cyanurated proteins were obtained after 2 min at pH 13.5, since it was found that the CyF-treated chymotrypsins developed turbidity on standing at that pH. The complete ionization of tyrosine residues in the chymotrypsins, however, requires 15–20 min. Therefore, it became necessary to correct the 2-min absorption values of the CyF-treated enzymes to 100% ionization of the unreacted groups. This was done with a correction factor, which was the degree of ionization attained by the untreated enzyme after 2-min exposure to pH 13.5. This value was determined each day in triplicate and found to be well reproducible.

Method of Data Analysis. The data were analyzed according to the previously described procedure (Gorbunoff, 1969). The discrete levels of tyrosine reactivity were established from the appearance of plateaus in the concentration curves. It seems worth stressing that the conditions chosen for running such concentration curves must be such that there should be no time-dependent tyrosine ionization, and that the ionization behavior should be identical both in the presence and absence of 10% dioxane (Gorbunoff, 1967–1969).

Enzyme Assays. The enzymatic activity of the chymotrypsins was determined spectrophotometrically at 256.0 nm using *N*-Bz-L-TyrOEt. After completion of the cyanuration reaction, modified samples of the enzyme were dialyzed overnight at 4° in 0.001 M HCl to remove bicarbonate buffer and excess of cyanuric acid. Three references were run with each experiment: (1) enzyme dissolved in 0.001 M HCl (standard A); (2) enzyme which had been taken through the cyanuration procedure without CyF, followed by the identical dialysis (standard B); and (3) enzyme which was mixed with the cyanuration mixture (the product of the cyanuration procedure from which protein had been absent) just prior to dialysis in 0.001 M HCl (standard C).

Acetylation with *N*-acetylimidazole was carried out at pH 7.5 according to the method of Riordan *et al.* (1965), except that chromatography on a Sephadex G-25 column was replaced by dialysis in 0.05 M pH 7.5 borate buffer.

Circular dichroism experiments were carried out on a Cary Model 60 spectropolarimeter as described previously (Gorbunoff, 1969).

Results

Titration. All three enzymes were titrated spectrophotometrically between pH 8.5 and 13.5 in 1 M $KHCO_3$ buffer, both in the presence and absence of 10% dioxane. The results obtained with α-chymotrypsin were in good agreement with data previously reported below pH 12.5 (Havsteen and Hess, 1962; Marini and Wunsch, 1963; Inada *et al.*, 1964). The ionization became time dependent above pH 12; thus, at pH 13.5, 3.2 tyrosines are ionized at 2 min and 3.4 at 5 min. The maximum in the difference spectrum was found to be at 294 nm below pH 12. Above this pH it shifted gradually, reaching 298 nm at pH 13.2, where a second maximum appeared at 290 nm and a minimum at 292 nm as previously reported by Inada *et al.* (1964). Addition of 10% dioxane had no effect below pH 12.5. Above this pH, it enhanced the ionization to a small extent, reflecting a slight destabilization of the protein molecule.

The results obtained with δ- and γ-chymotrypsins at 298 nm are shown in Figure 1. Similar results were obtained using the 245-nm data. The two titration curves in the buffer are identical up to pH 11.3; above this pH, γ-chymotrypsin is more resistant to ionization. The data can be described best in terms of both proteins possessing two tyrosine residues with a pK_{app} of 10.5; the third group has a pK_{app} of 11.9 in δ and of 12.4 in γ. The fourth group is highly resistant to ionization in both forms of the enzyme. The titration becomes time dependent in both proteins above pH 12, with γ remaining less ionizable. At pH 13.5, 3.2 groups ionize in δ after 2 min and 3.9 after 30 min. In γ, the corresponding

TABLE I: Preliminary Cyanuration Experiments.

				4°				
pH		9.4	10.0	10.5	11.0	11.5	12.0	12.5
Average number of	α:	0.8	1.0	1.4	1.5	1.8	2.0	2.3
tyrosines reacted	δ:	1.0	1.3	1.7	1.9	2.0	2.0	
	γ:	1.0	1.5	2.0	2.1	2.6		
				25°				
Average number of	α:	1.1	1.9	2.4	2.7	3.4	3.1	
tyrosines reacted	δ:	2.0	2.2	2.5	3.0	3.4	3.4	
	γ:	2.0	2.6	3.0	3.4	3.3		

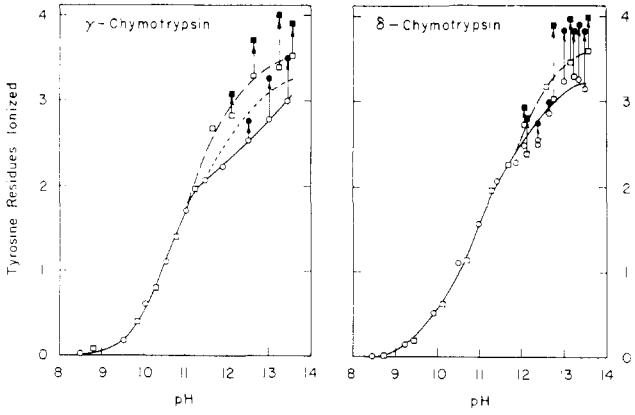

FIGURE 1: Tyrosine titration curves of δ- and γ-chymotrypsins in 1 M $KHCO_3$ buffer. Circles: without dioxane; squares: with 10% dioxane; open symbols: 2 min; filled symbols: 30 min.

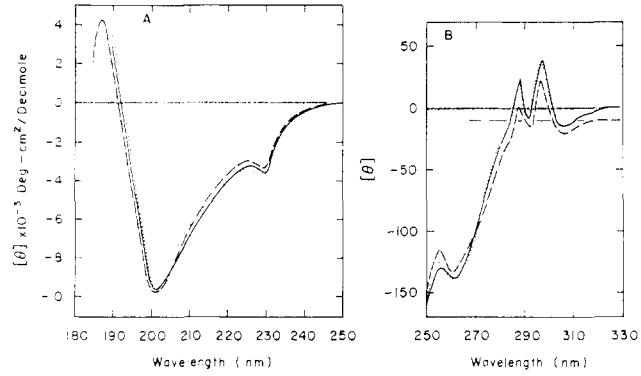

FIGURE 2: Circular dichroism of the chymotrypsins in 0.001 M HCl. Solid line: α-chymotrypsin; dashed line: γ-chymotrypsin; dotted line: δ-chymotrypsin. In part B, the spectrum of γ-chymotrypsin has been displaced arbitrarily by $-10[\theta]$.

numbers are 3.0 and 3.5. The maximum in the difference spectra was at 295 nm below pH 12 in both proteins. Above this pH, this maximum shifted gradually to 298 nm and a second maximum (at 290 nm in δ and 291.5 nm in γ) and a minimum (at 293 nm in δ and 293.5 nm in γ) slowly appeared (in δ, this takes 20 min at pH 13.1).

Addition of 10% dioxane has no effect on the tyrosine titration of γ-chymotrypsin below pH 11.2 and of the δ enzyme below pH 12. Above these pH's, 10% dioxane exerts a significant destabilization on both enzymes. In γ-chymotrypsin, it leads to the ionization of about one-half additional residues after 2 min, while in δ the effect is smaller; in fact, the 2-min curves of γ- and δ-chymotrypsins in 10% dioxane are essentially identical. Standing for 30 min in this medium above pH 12.7 leads to full ionization of the four residues in the δ enzyme. The fourth residue also becomes fully ionized in δ-chymotrypsin on standing 30 min above pH 13 in the buffer without dioxane while it is still quite resistant to ionization in the γ enzyme at the same conditions.

Circular Dichroism. In view of the subtle differences in tyrosine titration of the three enzymes, all three were examined by circular dichroism spectroscopy between 340 and 190 nm. The results, shown in Figure 2, indicate the absence of gross structural differences between the three forms, in agreement with the conclusions of X-ray crystallography. All three spectra are characterized in the far-ultraviolet region by a negative maximum at 202 nm and positive ellipticity below 193 nm. The negative maximum at 230 nm and minimum at 222 nm, which are related to the activation process (Hess, 1969), are present in all three forms. In the near-ultraviolet region, all three enzymes display circular dichroism spectra with positive maxima at 288 and 297 nm and negative extrema at 291 and 305 nm, as well as a negative extremum at 262 nm and a minimum at 255 nm.

Cyanuration. Preliminary experiments were carried out at a CyF concentration of 0.023 M[1] at 25° and 4° in the pH range below the conformational destabilization induced by 10% dioxane. All three enzymes were reacted at pH 9.4, 10.0, 10.5, 11.0, and 11.5; δ-chymotrypsin was also cyanurated at pH 12.0, and the α enzyme at pH 12.0 and 12.5. The results are compiled in Table I. As can be seen, for the α enzyme there is a constant difference of about one reacted residue between the two temperatures in the pH range of 10–12; for the δ and γ forms, this pH range is between 9.4 and 11. For δ-chymotrypsin, concentration curves were obtained at 4° at pH's 9.4, 11.0, 11.5, and 12.0 and at 25° at pH's 9.4 and 11.0. As shown on Figure 3, there is a plateau at the level of one residue at pH 9.4 at 4°; plateaus at the level of two residues are obtained at pH 9.4 and 25°, and at pH 11.0, 11.5, and 12.0 at 4°; a plateau of three residues is found at pH 11.0 and 25°.

In the case of γ-chymotrypsin, concentration curves were obtained at 4° at pH's 9.4, 10.5 and 11.0 and at 25° at pH's

[1] In previous papers (Gorbunoff, 1967–1970), the concentration of CyF reported was that of the reagent added to the final reaction mixture, according to Kurihara *et al.* (1963). In the present paper, the concentration is that found in the reaction mixture after addition of all components.

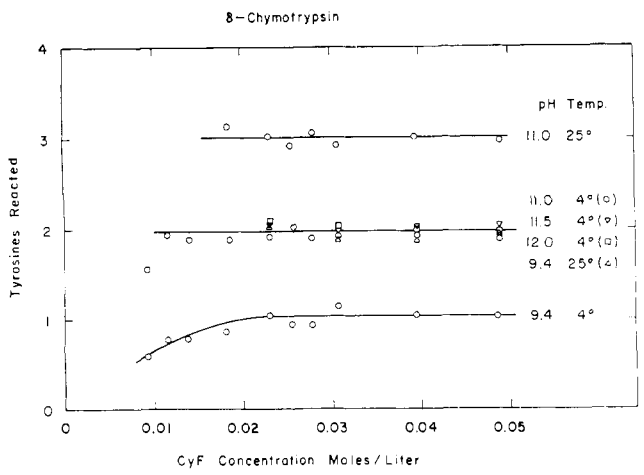

FIGURE 3: Dependence of δ-chymotrypsin tyrosine reactivity upon CyF concentration at 4 and 25° as a function of pH.

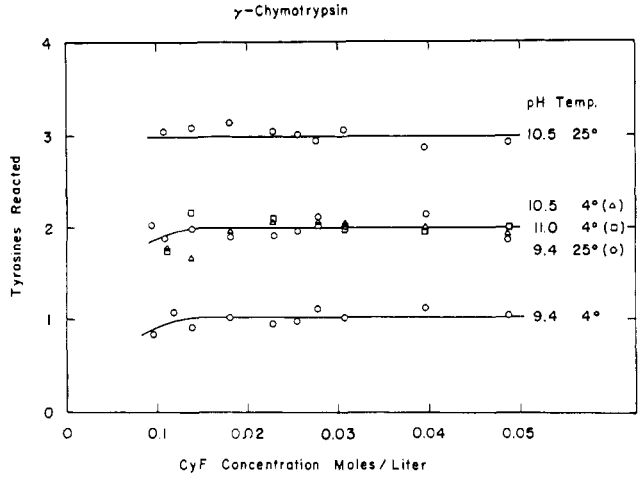

FIGURE 4: Dependence of γ-chymotrypsin tyrosine reactivity upon CyF concentration at 4 and 25° as a function of pH.

9.4 and 10.5. As shown on Figure 4, there is a plateau at the level of one residue at pH 9.4 at 4°; plateaus at the level of two residues were obtained at pH 9.4 at 25° and at pH 10.5 and 11.0 at 4°; a plateau of three residues was obtained at pH 10.5 at 25°.

For α-chymotrypsin, concentration curves were obtained at 4° at pH's 9.4, 10.0, and 11.5, and at 25° at pH's 10.0 and 11.0. As shown on Figure 5, there are plateaus at the level of one residue at pH's 9.4 and 10.0 at 4°; plateaus at the level of two residues were obtained at pH 10.0 at 25° and at pH 11.5 at 4°; a plateau of three residues was obtained at pH 11.0 at 25°. Furthermore, plateaus of two residues appear to be maintained at 4° at pH's 12.0 and 12.5, and of three residues at 25° at pH 12.0, as shown by identical extents of cyanuration at these conditions in 0.023 and 0.048 M CyF.

δ-Chymotrypsin was also acetylated with *N*-acetylimidazole over molar ratios of protein to reagent of 1:47 to 1:1200, resulting in the acetylation of 0.5–2.0 residues. This degree of acetylation did not affect significantly the protein conformation, since the circular dichroism spectrum for the diacetylated enzyme was found to be essentially identical with that of the native protein.

Activity. The effect of various degrees of cyanuration on the enzymatic activity was determined for the three enzymes. In preliminary control experiments, the enzymes were first exposed to the reaction conditions (*i.e.*, standing for 45 min in 1 M $KHCO_3$ buffer of the proper pH in 10% dioxane at 4 or 25°) in the absence (standard B) and presence (standard C) of the reaction product (cyanuric acid), followed by overnight dialysis. The results are summarized in Table II where the activities are given as per cent of the activity of the untreated enzyme.

In δ-chymotrypsin, exposure to the buffer alone between pH 9.4 and 11.0 resulted in about 20% loss of activity, both at 4 and 25°. The presence of cyanuric acid did not seem to affect the activity to a significant extent up to pH 10.5; at pH 11.0, it led to a 40% loss of activity.

In the α and γ enzymes, the situation is considerably different. Exposure to buffer leads to activity losses of 10–30% with the largest losses occurring at 25° at the highest pH's. Exposure to cyanuric acid, however, leads to a striking drop in activity. In α-chymotrypsin, this decrease is of the order of 30–50%; in γ-chymotrypsin, the activity loss varies between 42 and 78%, the decrease in activity being enhanced both by an increase in pH and temperature. Thus, while in δ-chymotrypsin cyanuric acid has no effect on activity, or possibly even exerts a slight protection, in the two forms of the enzyme from which the Thr-147-Asn-148 dipeptide had been cleaved out, contact with this compound induces a significant inhibition of enzymatic activity.

The three enzymes were then cyanurated to various extents and the effect on activity of covalently blocking the tyrosine hydroxyls by the triazine group was established. The results are reported in two ways in Table II. The per cent activity relative to the starting enzyme (Cy-Ct) is given in line four, while the last line is the activity relative to that of standard C, *i.e.*, to enzyme that had been subjected to the reaction conditions without the formation of new covalent bonds.

In δ-chymotrypsin, cyanuration at 4° at pH 9.4 and 11.0, where one and two tyrosines react specifically, resulted in no loss of activity relative to standard C. In order to examine the effect of the reaction temperature on the activity, the enzyme was modified to the extent of the equivalent of one and two tyrosine residues at pH 9.4 and 11.0 at 25°, selecting CyF concentrations which give average values of one and two reacted groups. In both cases, raising the reaction temperature resulted in a drop of activity. In α- and γ-chymotrypsins,

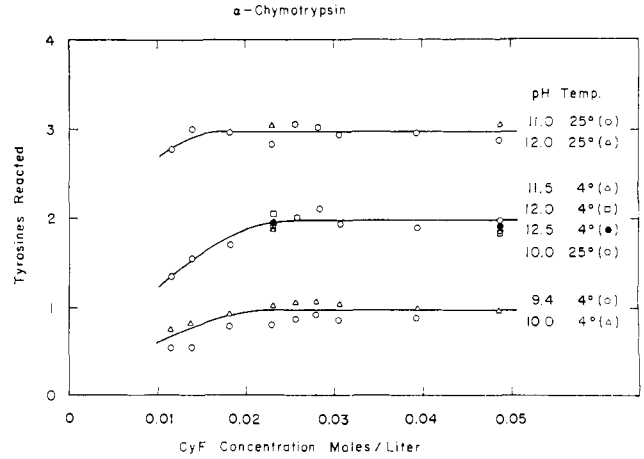

FIGURE 5: Dependence of α-chymotrypsin tyrosine reactivity upon CyF concentration at 4 and 25° as a function of pH.

TABLE II: Effect of Cyanuration on the Enzymatic Activities of the Chymotrypsins.[a]

	4°				25°		
			δ-Chymotrypsin				
pH	9.4	10.0	10.5	11.0	9.4	10.0	11.0
Groups reacted	1	(1)[b]	(1)	2	(1)	(2)	(2)
Standard B	76	80	80	76	81	78	82
Standard C	95	86	88	59	98	91	58
Cy-Ct	94	76	72	58	74	67	51
Cy-Ct–StC	99	89	82	99	75	73	89
			γ-Chymotrypsin				
pH	9.4	10.0	10.5		9.4	10.0	10.5
Groups reacted	1	(1)	2		2	(2)	3
Standard B	75	87	75		75	58	67
Standard C	53	50	36		58	40	22
Cy-Ct	44	48	26		30	22	10
Cy-Ct–StC	83	96	71		47	56	45
			α-Chymotrypsin				
pH	9.4	10.0			10.0	11.0	
Groups reacted	1	1			2	3	
Standard B	77	95			72	66	
Standard C	63	63			52	56	
Cy-Ct	60	47			26	16	
Cy-Ct–StC	95	73			51	29	

[a] All activities (except for last line) are reported as percentage of activity of chymotrypsin not exposed to reaction conditions. Last line is activity of cyanurated enzyme relative to standard exposed to full reaction conditions. [b] The numbers in parentheses represent effective degrees of cyanuration obtained by properly adjusting CyF concentration as described in the text.

progressive cyanuration led to a progressive loss of activity. Modification of one residue at pH 9.4 and 4° had little or no effect on the activity relative to standard C. Blocking of a second tyrosine reduced the activity to about 25% of that of the untreated enzyme, or 50–70% of the standard, in contrast to the case of the δ enzyme. Cyanuration of a third group resulted in a further decrease in activity in both the α and γ forms.

Discussion

The titration results suggest that the first two tyrosines of δ- and γ-chymotrypsins are equally accessible to hydrogen ions; the two enzymes titrate in identical manner in 1 M $KHCO_3$, with and without dioxane, up to pH 11.3. In this pH range, each displays two ionizable groups with pK_{app} of 10.5, although the data could be fitted equally well with two groups having slightly different pK's, one slightly lower and the other slightly higher. The third residue titrates less readily in the γ form (pK_{app} = 11.9 in δ and 12.4 in γ). The titration curve of α-chymotrypsin has been characterized in terms of three groups titrating with pK_{int} of 10.2, 11.3 and 12.5 (Inada *et al.*, 1964; Havsteen and Hess, 1962; Marini and Wunsch, 1963; Tanford, 1962). The fourth group is highly resistant to ionization in all three enzymes. Exposure to 10% dioxane and time effects, however, reveal a definite gradation; this residue is more fully ionized after 30-min standing above pH 13 in δ- than in γ-chymotrypsin. It is the least affected by dioxane in the α enzyme. These differences in titration properties indicate that subtle variations exist between the environments of the four groups in the three forms of chymotrypsin. These may be either structural or electrostatic. Crystallographic studies indicate that, while γ-chymotrypsin is chemically identical with α, conformationally it may be more closely related to the δ form. A combination of these two factors may lead to the observed differences in tyrosine ionizability. Since the conformational stabilization forces in proteins are cooperative in nature, it is quite likely that significant alterations in structures in one region may be translated into subtle variations in other regions of the molecule, as the free energy of stabilization is redistributed. Thus, while the chemical and crystallographic studies have revealed so far differences only in the environment of Tyr-146, these may be accompanied by much smaller, but on a short range, significant differences in the environments of the other tyrosyl residues.

The results of the cyanuration reaction display a similar picture from the point of view of another criterion of accessibility. In all three forms of the enzyme, only three tyrosine residues are accessible to this reagent, the degree of accessibility varying between the individual groups. Furthermore, it is quite striking that there is a consistent difference in tyrosine reactivity by one group between 4 and 25°. Brandts (1964) has shown that chymotrypsinogen is more stable at 4° than at 25°; the same is true of α-chymotrypsin (Biltonen and Lumry, 1965). Group by group comparison for the three enzymes shows that subtle differences exist between them. In general the tyrosines of γ-chymotrypsin appear to be the most reactive ones, while those of the α enzyme are the least reactive. The first group is indistinguishable in all three enzymes; it gives a plateau at 4° and pH 9.4 in all three forms; this plateau is maintained at pH 10.0 in α-chymo-

trypsin, while in the other two forms the second residue is beginning to react at that pH. The second group seems to be most accessible in the γ enzyme, giving a plateau in that protein at 4° and pH 10.5. In δ-chymotrypsin, the second plateau is obtained at pH 11.0 at the same temperature; at pH 10.5, the approach of a plateau is suggested by poorly reproducible results as a function of CyF concentration, indicating that the difference in accessibility of the second group is very small between the γ and δ enzymes. In α-chymotrypsin, the second plateau is not attained until pH 11.5 at 4°. A similar situation persists at 25°, where two group plateaus are obtained in the δ and γ enzymes at pH 9.4, and only at pH 10.0 in the α form. The third group is most reactive in γ-chymotrypsin, where it gives a plateau at pH 10.5 and 25°, while pH 11.0 is required in the α and δ enzymes.

Both the degrees of exposure to the cyanuration reaction and the titration curves were obtained over a pH range in which α- and δ-chymotrypsins are known to undergo a reversible conformational change. This change which occurs between pH 6 and 11, with a midpoint at pH 8.3 (Oppenheimer *et al.*, 1966), is known to be associated with the opening up of the Ile-16-Asp-194 ion pair. It may be assumed that the same structural conversion occurs also in γ-chymotrypsin. This transition may very well affect the immediate environments of the second and third residues. It need not affect that of the first one, provided that it is a completely accessible one. In fact, it would be highly surprising if it did not affect Tyr-146, due to the proximity of that group to the region of the molecule in which the structural rearrangement occurs. The decreased reactivity of the second residue in α-chymotrypsin relative to γ and δ merits some comment. At 4°, it gives a plateau only at pH 11.5, which is maintained up to pH 12.5; at 25° the three group plateau is maintained from pH 11 to 12. Havsteen and Hess (1962) have established pH 12 at 16° as the point above which irreversible conformational changes set in, in agreement with the earlier optical rotatory dispersion studies of Neurath *et al.* (1956). Thus, it appears that, in the α enzyme, accessibility of the second and third groups to CyF requires a considerable loosening of structure (Neurath *et al.*, 1956; Havsteen and Hess, 1963; Himoe *et al.*, 1967). The lack of increase in tyrosine reactivity in this pH region may simply indicate either that, at 4°, the conformational change does not yet set in or that the tyrosine cyanuration reaction, which is known to be very rapid (Gorbunoff, 1967), reaches completion before the residues in question become affected by a change in conformation. As a result, plateaus are maintained at the level of two groups in δ-chymotrypsin at pH 12 and 4° and in the α enzyme at pH 12.5 and 4°.

The results of the cyanuration and titration studies are fully consistent in that both show a gradation in group availability. The differences between the two most probably reflect differences in the criteria of accessibility of the two methods. The energy barriers in the two cases are controlled by different factors. Binding of hydrogen ions is most strongly affected by local electrostatic interactions (Tanford, 1957, 1962; Timasheff, 1970b), while reaction with CyF also has rigorous steric requirements (Gorbunoff, 1970).

The essential identity of the circular dichroism spectra of the three enzymes (Figure 2) serves to support the notion that the conformational differences between them are very subtle. In the region below 250 nm, all three have the band between 220 and 230 nm, suggesting that the Ile-16-Asp-194 ion pair is formed identically in all three. The lack of difference in the circular dichroism spectra at lower wavelengths supports the crystallographic conclusion that the secondary structures of the three are essentially identical. The decrease in intensity at the 200–204-nm peak from the zymogen (Gorbunoff, 1969) supports the conclusion drawn from nuclear magnetic resonance studies that the exact conformations of the zymogen and enzyme in solution are not identical (Hollis *et al.*, 1968; Lumry and Biltonen, 1969). In the near ultraviolet region, the spectra of the three enzymes are almost identical not only with each other but also with that of the zymogen. The circular dichroism spectrum of γ-chymotrypsin is less intense than those of the others between 250 and 275 nm, suggesting possibly slight differences in interactions of the corresponding chromophoric groups. Acetylation experiments both with the zymogen (Gorbunoff, 1969) and δ-chymotrypsin, suggest that tyrosines contribute little to that region of the circular dichroism spectrum. The positive bands at 288 and 297 nm and the negative band at 308 nm are best assigned to tryptophan transitions (Gorbunoff, 1969; Strickland *et al.*, 1969; Timasheff, 1970a), while the detail between 250 and 270 nm is probably due to phenylalanine residues (Horwitz *et al.*, 1969); disulfides may contribute both to that spectral region and to the negative absorption between 300 and 320 nm (Beychok, 1967; Coleman and Blout, 1968).

At this point it seems of interest to compare the present results with what is known about the states of tyrosines in the chymotrypsins. The literature on this subject in the chymotrypsin family is very limited. Two tyrosine residues can be acetylated with *N*-acetylimidazole in α-chymotrypsin at a 180-fold molar ratio of reagent to protein (Riordan *et al.*, 1965). The same number of tyrosines could be acetylated in δ-chymotrypsin in the present studies at a protein to reagent molar ratio of 1:300. The acetylation of α-chymotrypsin can be carried, however, to the limit of four groups if a very large excess of *N*-acetylimidazole is used (Gorbunoff, 1969). Iodination studies of α-chymotrypsin (Dube *et al.*, 1964, 1966) showed that even at the level of less than 1 g-atom of iodine introduced per mole of protein, Tyr-146, -171, and -94 are iodinated, with preferential iodination of Tyr-146. Hachimori *et al.* (1965) have reported the cyanuration of two tyrosine residues, while Shlyapnikov *et al.* (1968) were able to nitrate Tyr-146 and -171 in the same enzyme. The results of modification with CyF cannot be compared without reservations to modification results obtained in iodination or nitration studies. Since the accessibility of a residue to a reagent is a function of the method of probing, chemical modification studies can be expected to give identical results only when the modification affects the same position on the residue within the restrictions imposed by reaction mechanisms and the geometric requirements of transition state complexes (Gorbunoff, 1970). Since nitration with trinitromethane and iodination attack the benzene ring of the tyrosine, while CyF attacks its oxygen atom, accessibility to the reagent does not have the same meaning for these two types of modifiers. This thesis is supported by the calculations of Lee *et al.* (1970), who showed that, in the case of ribonuclease S, the tyrosine hydroxyls and the ring carbons are exposed to nonidentical extents.

The only unequivocal evidence on the difference in the states of tyrosine residues within the chymotrypsin family is restricted to Tyr-146 and comes from the X-ray studies. Its application to the present work in solution is complicated by the fact that α-chymotrypsin exists as a dimer in the crystal, with the consequence that Tyr-146 becomes buried. In the crystal this group can be only monoiodinated, while it forms

the diiodo derivative in solution. In the γ form, iodination is identical in the two states of dispersion, giving diiodotyrosine. The difference in Tyr-146 conformation between the α and γ enzymes in the crystal seems to be carried over to the solution state, since α-chymotrypsin can undergo a dimerization reaction below pH 5.5, while the γ enzyme dimerizes only very weakly, if at all (Schwert, 1949; Aune and Timasheff, 1971). A further complication arises from the fact that crystallization is carried out in the pH range below neutrality, while the present study concerns itself with the alkaline pH region within a zone over which the enzyme undergoes a conformational transformation. Since the ability to dimerize at low pH and the crystallographic properties of the chymotrypsins are related (Wright *et al.*, 1968; Sigler *et al.*, 1968), one must conclude that conformationally Tyr-146 of the γ enzyme is similar to π- and δ-chymotrypsins and not to the α analog. On the other hand, there should be differences between the states of the electrostatic environment of Tyr-146 in π- and δ-chymotrypsin and that of the α and γ enzymes, since the latter carry an ionized α carboxyl on Tyr-146, while the former do not. Thus, one would expect small, but nevertheless real, differences between the environment and accessibility of Tyr-146 in the three enzymes. Such differences are displayed best by the second plateau.

The effect of the cyanuration reaction on biological activity of the chymotrypsin family is summarized in Table II. The effect on enzyme activity of exposure to 1 M $KHCO_3$ buffer, containing 10% dioxane, does not differ greatly between the three enzymes. Introduction of cyanuric acid, followed by dialysis, however, leads to a dramatic difference between the activities of the enzymes. δ-Chymotrypsin is essentially not affected by cyanuric acid; the loss of activity varies between 2 and 12% at pH's 9.0, 10.0, and 10.5 at 4 and 25°; at pH 11.0, however, this loss rises to 42%. α and γ, on the other hand, are affected very strongly; α retains 50–60% of its original activity, while in γ, the activity drop reaches 78%. While this difference in the inhibitory effect of minute amounts of cyanuric acid is unexplained at present, it seems to be related to whether Tyr-146 exists as a C-terminal residue or as part of the chain. Cyanuric acid may be expected to be a strong chelating agent, as a result of its aromatic ring and three acidic hydroxyls. Indeed in experiments with other proteins, it has been found impossible to remove it completely by dialysis (M. J. Gorbunoff, unpublished data).

The effect of tyrosine cyanuration upon biological activity is shown under Cy-Ct and Cy-Ct-standard C. Modification of one or two tyrosines in δ-chymotrypsin has no effect on biological activity, in agreement with the retention of activity by this form when two tyrosines are acetylated (Oppenheimer *et al.*, 1966; Karibian *et al.*, 1968). Modification of one tyrosine residue in the α and γ enzymes does not affect activity relative to standard C. Reaction of the second tyrosine in the α and γ enzymes is accompanied by an activity loss. Cyanuration of the third tyrosine residue leads to almost complete loss of activity. Nevertheless, it does not appear justifiable to relate the loss of activity solely to tyrosine residue modification, considering that the loss of activity brought about by the presence of cyanuric acid (standard C) is already quite high. Therefore, the difference in the behavior between the δ enzyme and the α and γ analogs must be due, at least in part, to the ability of the latter two to interact with cyanuric acid at some specific center, which is not present in the δ enzyme. This difference in binding of cyanuric acid may be related to the known difference in binding of competitive inhibitors by the α and δ enzymes (Valenzuela and Bender, 1970). The interaction with cyanuric acid seems to be strongest in the γ enzyme and to increase in it with increasing temperature and pH. Thus, in general, the γ enzyme seems to be the most labile of the three.

While the activation process is not accompanied by any gross structural changes (Matthews *et al.*, 1967, 1968; Kraut *et al.*, 1967; Sigler *et al.*, 1968; Wright *et al.*, 1968; Freer *et al.*, 1970; Krigbaum and Godwin, 1968), small changes are suggested by nuclear magnetic resonance studies (Hollis *et al.*, 1968), X-ray studies (Freer *et al.*, 1970), and the present circular dichroism data. The environment of some tyrosine residues must be affected since the chymotrypsinogen–chymotrypsin difference spectrum shows a strong band at 287 nm (Chervenka, 1957, 1959; Benmouyal and Trowbridge, 1966). Titration studies (Tanford, 1962; Havsteen and Hess, 1962; Inada *et al.*, 1964) indicate for both proteins the presence of two reversibly titratable tyrosines with different pK's; there are two buried tyrosine residues. The present work with CyF provides support for the occurrence of significant changes in the tyrosine residue environment during the activation process. It has been shown earlier (Gorbunoff, 1969) that chymotrypsinogen has two CyF reactive residues. The first residue is accessible to this reagent in the native state at 4° and pH 10.5, while the second becomes accessible only after the onset of irreversible denaturation at 25° and pH 12. Thus, activation liberates one additional tyrosine residue to reaction with CyF. Furthermore, the CyF-reactive residues of the zymogen are less reactive than those in the enzyme. This is attested to by the more rigorous conditions required for their cyanuration. The difference between the zymogen and the δ enzyme, in both of which Tyr-146 is not terminal, seems particularly interesting.

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