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Arsanilazochymotrypsinogen. The Extrinsic Cotton Effects of an Arsanilazotyrosyl Chromophore as a Conformation Probe of Zymogen Activation*

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ABSTRACT: Chymotrypsinogen A modified with diazotized *p*-arsanilic acid exhibits a characteristic circular dichroic spectrum with a small positive Cotton effect at 323 nm and a large negative one centered at 428 nm with a molecular ellipticity, $[\theta]^{26}_{428}$, of $-8000°$, characteristic of azo aromatic modification. Exposure of 6.4×10^{-5} M arsanilazochymotrypsinogen to concentrations of trypsin ranging from 1.9×10^{-7} to 7.6×10^{-7} M at pH 7.5, inverts the negative sign of the long-wavelength Cotton effect; the positive band, now centered at 448 nm, has a molecular ellipticity, $[\theta]^{26}_{448}$, of $+7000°$. The changes in sign, position, and magnitude are first order with respect to arsanilazochymotrypsinogen concentration and display pseudo-first-order rate constants directly proportional to the trypsin concentration. Both native and arsanilazochymotrypsinogens are activated by trypsin at similar rates, and the resultant enzymes display nearly identical activities toward *N*-benzoyl-L-tyrosine ethyl ester.

The variation in optical activity correlates closely with the induction of enzymatic activity, as gauged by the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester, and seemingly reflects the kinetics of structural rearrangements accompanying zymogen activation. The extrinsic Cotton effect of the arsanilazo aromatic chromophore provides a sensitive signal of changes in local conformation which accompany activation of the modified chymotrypsinogen.

In the process of zymogen activation rearrangements of protein structure have been thought to result in the juxtaposition of amino acid side chains critical for effective substrate binding and catalysis (Neurath, 1964). However, the experimental demonstration of such postulated conformational changes has proven difficult. The activation of bovine chymotrypsinogen A has been examined in particular detail in this regard. Hydrolysis of the Arg-15-Ile-16 bond is both necessary and sufficient to generate enzymatic activity (Jacobsen, 1947; Dreyer and Neurath, 1955; Hartley and

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Kauffman, 1966; Meloun *et al.*, 1966). X-Ray diffraction studies of chymotrypsinogen A and α-chymotrypsin as well as other active products of this zymogen suggest that the overall folding of their peptide chains is very similar, and major rearrangements of the protein structure consequent to activation are not apparent (Kraut *et al.*, 1967; Sigler *et al.*, 1968; Cohen *et al.*, 1969; Freer *et al.*, 1970). A "charge-relay system," thought to account for the enhanced chemical reactivity of serine-195 subsequent to activation (Blow *et al.*, 1969), appears to be present also in the zymogen (Freer *et al.*, 1970). Studies by means of a variety of experimental techniques, *e.g.*, absorption (Chervenka, 1959), optical rotatory dispersion, and circular dichroism spectroscopy (Neurath *et al.*, 1956; Imahori *et al.*, 1960; Raval and Schellman, 1965; Biltonen *et al.*, 1965; Fasman *et al.*, 1966; McConn *et al.*, 1969), and hydrogen isotope exchange measurements (Willumsen, 1968) have led to various suggestions regarding alterations in the environments of some peptide bonds and aromatic residues consequent to activation. X-Ray diffraction studies are consistent with the postulate that *localized* conformational changes may occur (Freer *et al.*, 1970).

We have recently reported that covalent modifications of a variety of proteins with diazonium salts generate extrinsic Cotton effects in the 300- to 600-nm region of the spectrum. The resultant circular dichroic spectra are distinctive for each of the different proteins examined, seemingly reflecting characteristic, native conformations (Fairclough and Vallee, 1970). Such findings suggested that the circular dichroic properties of arsanilazozymogen–enzyme pairs could be employed to detect conformational changes which might accompany activation. The extensive and detailed knowledge of the chymotrypsinogen–chymotrypsin pair and the lack of major changes in primary structure during the activation process suggested that this system might be particularly revealing regarding the potential of optically active azo probes. The data show that the transformation of the circular dichroic properties of arsanilazochymotrypsinogen into those of the corresponding enzyme can, indeed, reflect subtle changes in protein structure and seemingly monitor the kinetics of structural rearrangements, amplifying events which are critical to the formation of the active enzyme. A preliminary report has been given (Fairclough and Vallee, 1969).

Materials

Lyophilized chymotrypsinogen A (CGC 8HA), α-chymotrypsin (CDI 6163), δ-chymotrypsin (CDD 6032), and trypsin (TRL 9DA) were purchased from the Worthington Biochemical Corp. *p*-Arsanilic acid was obtained from Eastman Chemicals. *N*-Benzoyl-L-tyrosine ethyl ester (lot U2315), L-1-tosylamide-2-phenylethyl chloromethyl ketone (lot 2224), and guanidine hydrochloride (Ultra Pure grade) were products from Mann Research Laboratories. All other chemicals were either reagent grade or the best grade available. Bio-Gel P-6 (50–100 mesh) was obtained from Bio-Rad Laboratories.

Methods

Diazotization of 0.50 mmole of *p*-arsanilic acid was carried out in 20 ml of 0.15 M HCl at 0° by slowly adding 0.55 mmole of sodium nitrite with continual stirring. After 15-min incubation, the pH of the solution was adjusted to 5.5 by careful addition of 3.4 M NaOH. This time period was sufficient for maximal diazotization of the *p*-arsanilic acid, as judged by the reaction of the fresh diazonium salt with phenol. The volume of the solution was adjusted to 25 ml by addition of ice-cold distilled water, resulting in a final reagent concentration of 0.02 M. This reagent preparation was used immediately for the coupling reaction.

Chemical modifications of chymotrypsinogen A and chymotrypsin were performed in 0.5 M sodium bicarbonate buffer solutions of varying pH's at a final protein concentration of 2×10^{-4} M. Suitable addition of diazotized *p*-arsanilic acid (0.02 M) produced a final reagent concentration of 4 mM. The reaction was terminated by the addition of sufficient 0.10 M phenol to react with excess reagent. The reaction mixture was then dialyzed exhaustively against 1 mM HCl at 4°. For the examination of the effects of pH upon modification, the protein was separated from the reaction mixture by gel filtration on Bio-Gel P-6, using 0.10 M NaCl–0.05 M Tris buffer at pH 7.5 as eluent.

Chymotryptic activity was determined using *N*-benzoyl-L-tyrosine ethyl ester (4.5×10^{-4} M, pH 7.8, 25°) in 0.05 M Tris buffer, 0.05 M $CaCl_2$, and 25% (w/w) methanol, as suggested by Hummel (1959). Changes in absorbance at 256 nm were recorded employing a Gilford Model 220 optical density converter and a Beckman DU monochromator with a thermostated cell compartment. A value of 964 cm^{-1} M^{-1} for the increase in absorbance produced by the hydrolysis of substrate was employed in calculating esterolytic activity.

Protein concentrations of unmodified chymotrypsinogen A and chymotrypsin were determined by absorbance at 282 nm assuming a specific absorptivity of 2.00×10^3 cm^{-1} g^{-1} ml (Wilcox *et al.*, 1957). A molecular weight of 25,700 was used for further calculations of chymotrypsinogen A concentrations (Tanford, 1968). Concentrations of the modified proteins were determined by the method of Lowry *et al.* (1951), altered according to Leggett-Bailey (1967), and employing the native enzyme or zymogen as standards. Chemical modification did not appear to affect the color yield. Further, the results of amino acid analysis agreed well with the amounts of protein calculated. The concentration of trypsin was determined by its absorbance at 280 mμ, using a specific absorptivity of 1.56×10^3 cm^{-1} g^{-1} ml and a molecular weight of 23,700 (Tanford, 1968).

Amino acid analyses were performed with a Spinco 120C amino acid analyzer. Acid hydrolysis of protein samples was carried out in sealed, evacuated tubes with 6 N HCl at 110° for 22 hr. *Arsenic analyses* were carried out on samples dialyzed exhaustively against 1 mM HCl using atomic absorption spectroscopy employing a 93-cm absorption cell according to the method of Ando *et al.* (1969) and an arsenic trioxide standard. Native chymotrypsinogen A did not affect the accuracy of the determination of added arsenic trioxide salts or *p*-arsanilic acid by this method.

Absorption spectra were obtained with a Cary 15 MS recording spectrophotometer, while absorption measurements at single wavelengths were performed with a Beckman DU. pH was determined with a Radiometer Model 25 pH meter, fitted with a scale expander using a GK2321C combination glass electrode.

Monoazotyrosyl and *monoazohistidyl* contents were evaluated from the absorption spectra of arsanilazo proteins in alkali, based on molar absorptivities proposed for the measurement of tyrosine and histidine modification (Tabachnick and Sobotka, 1959, 1960).

Circular dichroic spectra were measured with a Cary 60

TABLE I: Residue Modification and Arsenic Incorporation after Coupling Chymotrypsinogen A with Diazotized *p*-Arsanilic Acid.[a]

Residue	Amino Acid Anal.[b]	Spectral Anal.[c]	Total as Incorpd[d]
Tyrosine (moles/mole)	1.2 ± 0.2	0.9 ± 0.1	
Histidine (mole/mole)	<0.1	~0.1	
Lysine (moles/mole)	3.8 ± 0.3		
Arsenic (g-atoms)			1.2 ± 0.1

[a] Incubation of the zymogen (2×10^{-4} M) with diazotized *p*-arsanilic acid (4×10^{-3} M) was carried out for 45 min at pH 8.5, 0°, in 0.5 M bicarbonate buffer and was terminated by the addition of phenol. The modified protein was separated from other components of the reaction mixture by dialysis against 0.001 N HCl at 4° prior to characterization. [b] Evaluated from the amino acid composition of native minus that of modified chymotrypsinogen, each determined in triplicate analyses and based on 22 alanines/mole of native zymogen. There was no change in the composition of any of the other amino acids. [c] Azochromophore formation was evaluated from the molar absorptivity values at 460 and 500 nm for the modified zymogen in alkaline as described by Tabachnick and Sobotka (1960). Lysine modification is not detected by this method. [d] By atomic absorption spectrophotometry.

spectropolarimeter fitted with a Cary 6001 circular dichroism accessory as previously described (Kagan and Vallee, 1969; Fairclough and Vallee, 1970). Molecular ellipticity values are expressed in units of (deg cm^2) per dmole of protein. No correction for the refractive index of the solvent was made.

Zymogen activation was carried out at pH 7.5, 26° in 0.10 M NaCl–0.05 M Tris buffer by incubating active or modified chymotrypsinogen at concentrations of 1×10^{-5} to 1×10^{-4} M, with trypsin at concentrations of 0.1–0.8 μM. Changes in absorption and circular dichroism were monitored directly in thermostated cell compartments during the course of activation. The appearance of enzymatic activity was determined by halting the activation process after desired time intervals by diluting aliquots of the activation mixture 250-fold and then assaying for the amount of *N*-benzoyl-L-tyrosine ethyl esterase activity generated.

Results

Diazonium salts readily couple with proteins to form colored, optically active derivatives which exhibit distinctive, extrinsic Cotton effects (Fairclough and Vallee, 1970). In order to study the effects of zymogen activation on such circular dichroic spectra, we have sought mild conditions for coupling a minimal number of chymotrypsinogen side chains with diazotized *p*-arsanilic acid while inducing maximal circular dichroic changes. Optimal modification occurred upon treatment of chymotrypsinogen A (2×10^{-4} M) with a 20-fold molar excess of reagent at pH 8.5, 0°, for 45 min in 0.5 M sodium bicarbonate buffer.

Tyrosyl, histidyl, and lysyl residues are modified most readily (Tabachnik and Sobotka, 1959, 1960). Following exhaustive dialysis against 1 mM HCl, atomic absorption spectroscopy detects 1.2 g-atoms of arsenic/mole of modified zymogen. Absorption spectra in alkali demonstrate the formation of 0.9 monoazotyrosyl and 0.1 histidyl residue(s) per zymogen molecule. Comparison of the amino acid compositions of the native and modified zymogens reveals the loss of 1.2 tyrosines and 3.8 lysines but negligible amounts of histidine (Table I).[1] The absorption spectrum of the final product is similar to that described for other arsanilazoproteins and does not reveal unusual, distinctive features. The circular dichroic spectrum is distinctive and different from those of all other arsanilazoproteins examined previously (Kagan and Vallee, 1969; Fairclough and Vallee, 1970). There is a small, positive Cotton effect near 323 nm which coincides with the absorption maximum as well as a prominent, negative band at 428 nm where the absorption spectrum is featureless. The molecular ellipticity at 428 nm, $[\theta]^{26}_{428}$, is −8000° (Figure 1A,B).

To examine the effect of coupling on the catalytic potential of the zymogen both the native and modified zymogen (4.5×10^{-5} M) were exposed to trypsin (0.29 μM, pH 7.5, 25°) in 0.10 M NaCl–0.05 M Tris buffer. These conditions are similar to those previously described for "rapid" activation of chymotrypsinogen yielding first π- and then δ-chymotrypsin (Dryer and Neurath, 1955). Chemical modification neither significantly alters the rate of appearance of activity toward *N*-benzoyl-L-tyrosine ethyl ester nor the specific activity of the product of activation (Figure 2). Further, similar kinetic constants were obtained for the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester over the concentration range of 1×10^{-4} to 2×10^{-3} M. Values for V_{max} of 1.6×10^2 and 1.5×10^2 μmoles min^{-1} mg^{-1} and for K_m of 1.0×10^{-3} and 1.1×10^{-3} M were found for the products of activation of the native and chemically modified zymogens, respectively. The residues of chymotrypsinogen A, which are modified with diazotized *p*-arsanilic acid, do not seem critical either to the activation of chymotrypsinogen or to the activity of the resultant enzyme toward *N*-benzoyl-L-tyrosine ethyl ester.

The absorption spectrum of the arsanilazozymogen undergoes minimal alteration on addition of trypsin. The maximum at 323 nm shifts toward shorter wavelengths by 1–2 nm, and the absorptivity decreases slightly. In contrast, the circular dichroic spectrum of the arsanilazozymogen changes drastically: the negative extremum at 428 nm, $\theta^{26}_{428} = -8000°$, becomes positive and shifts 20 to 448 nm, $\theta^{26}_{428} = +7000°$, a total change of 15,000° (Figure 3). Such changes following the addition of trypsin (0.3 μM) cease after 60 min, and a second addition of fresh trypsin does not

[1] The chromatogram of the hydrolysate of the arsanilazozymogen reveals an anomalous peak preceding that of tyrosine and equal to about one-half of its area. This peak is similar to those in the hydrolysates of all other proteins modified with diazotized *p*-arsanilic acid examined by us to date. Its identity is currently unknown, but is not observed upon chromatography of the hydrolysate of the arsanilazo-derivative of *N*-acetyl-L-tyrosinamide. Nor does it seem to be identical with α-amino-ε-hydroxycaproic acid, which elutes between glycine and proline and which is a likely hydrolysis product of lysine, when modified in this manner. Characterization of this peak is in progress. The method of Tabachnick and Sobotka (1960) is based on properties of the mono-arsanilazo derivatives of *N*-chloroacetyltyrosine and *N*-acetylhistidine and presumes an identity with analogous chromophores generated in proteins. When modification is minimal, as in the present instance, differences between evaluations by spectral and amino acid analyses, similar to those observed here, are commonly observed.

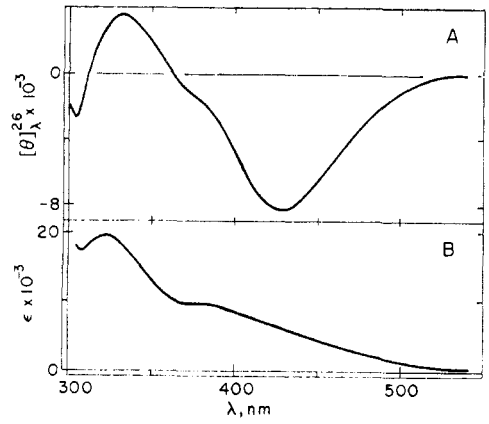

FIGURE 1: Circular dichroic (A) and absorption (B) spectra of arsanilazochymotrypsinogen in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5), 26°. The arsanilazozymogen was prepared by incubating zymogen (2×10^{-4} M) with diazotized *p*-arsanilic acid (4×10^{-3} M) for 45 min at pH 8.5, 0°, in 0.5 M bicarbonate buffer. The reaction was terminated by addition of phenol, and the protein was separated from other components of the reaction mixture by dialysis against 0.001 N HCl, 4°, prior to characterization.

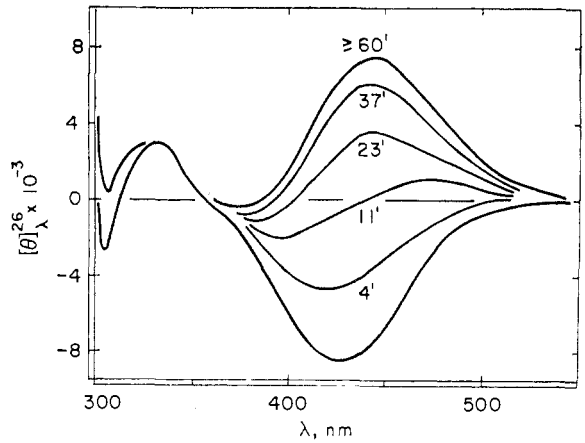

FIGURE 3: Circular dichroism of arsanilazochymotrypsinogen A before and during incubation with trypsin (2.9×10^{-7} M) in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5), 26°. The numbers indicate minutes elapsed between trypsin addition and the recording of a given circular dichroism measurement at 440 nm. The arsanilazozymogen was prepared as in Figure 1.

further alter the circular dichroic spectra which are also independent of the arsanilazochymotrypsinogen concentration over a range from 0.3 to 3.0 mg per ml.

Incubation of chymotrypsinogen with chymotrypsin results in enzymatically inactive neochymotrypsinogens (Rovery *et al.*, 1957). Hence, arsanilazochymotrypsinogen (5×10^{-5} M) was incubated with fully active α-chymotrypsin (5×10^{-5} M) to generate arsanilazoneochymotrypsinogens. After 60 min, 25°, the molecular ellipticity of the negative extremum at 428 nm varied by less than 6%. Thus, chymotryptic attack of the arsanilazozymogen does not invert the sign of the negative Cotton effect at 428 nm. Apparently such an inversion depends on tryptic cleavage.

Several approaches may be employed to indicate the presumable changes in primary structure which could account for the inversion of the Cotton effect at 428 nm. Alterations in the ratio of trypsin to arsanilazochymotrypsinogen were examined for possible effects on the sign and amplitude of this band. Molar concentrations of trypsin varying from 0.19 to 0.76 μM gave changes in circular dichroism, monitored continuously at 440 nm, which fit a first-order rate expression for 90% of the total change (Figure 4). These changes are directly proportional to the trypsin concentrations employed (Figure 4 and Table II), but are independent of initial arsanilazozymogen concentrations when these were varied from 1×10^{-5} to 1×10^{-4} M. Features, considered typical of autocatalysis, are not apparent in any of the progression curves. The results here obtained are to be expected if the attack of trypsin on the arsanilazozymogen is the specific, rate-limiting event which generates the circular dichroic changes.

The rate of appearance of activity toward *N*-benzoyl-L-

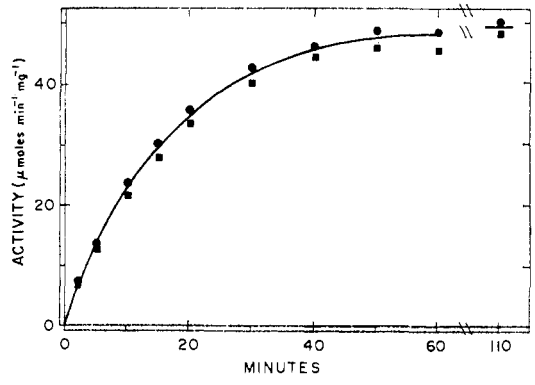

FIGURE 2: Generation of *N*-benzoyl-L-tyrosine ethyl esterase activity upon trypsin activation of native (●) and arsanilazochymotrypsinogen (■). Activation of zymogen (4.5×10^{-5} M) with trypsin (2.9×10^{-7} M) was performed at pH 7.5, 26°. Esterase activity was measured with *N*-benzoyl-L-tyrosine ethyl ester (4.5×10^{-4} M), pH 7.8, 25°. The arsanilazozymogen was prepared as in Figure 1.

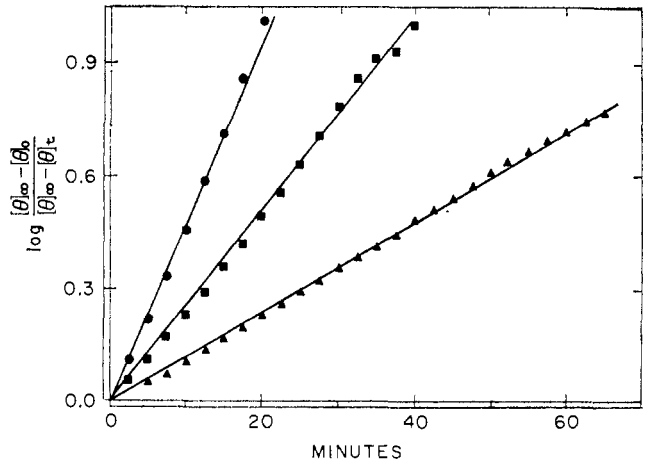

FIGURE 4: First-order rate plots of changes in circular dichroism upon trypsin activation of arsanilazochymotrypsinogen A. The arsanilazozymogen (6.4×10^{-5} M) was incubated with trypsin at concentrations of 1.9×10^{-7} (▲), 3.8×10^{-7} (■), or 7.6×10^{-7} M (●—●) in 0.10 M NaCl–0.05 M Tris buffer, pH 7.5, 26°. The course of the change in circular dichroism was recorded directly at 440 nm, the wavelength of the maximal difference in the optical activity of the arsanilazozymogen and its product of activation. From such curves, values were calculated for the molecular ellipticity at 440 nm observed initially, $[\theta]_0$, after desired periods of incubation, $[\theta]_t$, and upon completion of the activation, $[\theta]_\infty$. These values were employed to construct the rate plots.

TABLE II: Effect of Variation in the Initial Concentrations of Reactants on the Observed Rate Constants for the Change in Optical Activity upon Activation.[a]

Trypsin (M × 10^7)	Azozymogen (M × 10^5)	k (min^{-1})	$\frac{k\ (min^{-1})}{\text{Trypsin (M)}} \times 10^{-5}$
1.9	6.4	0.028	1.5
3.8	1.6	0.051	1.3
3.8	3.2	0.054	1.4
3.8	6.4	0.058	1.5
3.8	12.8	0.052	1.4
7.6	6.4	0.108	1.4

[a] Activation conditions: 0.10 M NaCl–0.05 M Tris buffer, pH 7.5, 26°.

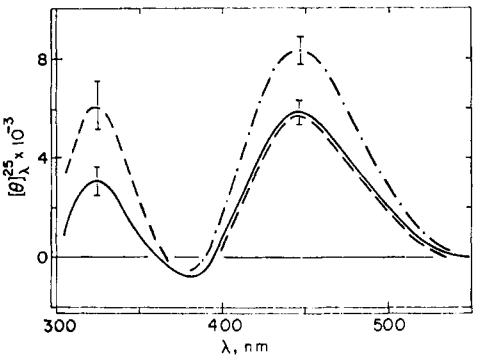

FIGURE 6: Circular dichroism of arsanilazochymotrypsins derived from δ-chymotrypsin (——) and α-chymotrypsin (- - - -) and the effect of 2.5 × 10^{-4} M L-1-tosylamido-2-phenylethyl chloromethyl ketone (·····). Protein modification and sample preparation as described in Figure 1.

tyrosine ethyl ester and that of the alterations in circular dichroism between 428 and 448 nm correlate closely, as measured on separate aliquots of the incubation mixture (Figure 5). Apparently, the alterations of the circular dichroic properties of the arsanilazochromophore reflect events accompanying zymogen activation.

In order to permit comparisons of the properties of product of activation with those of the two enzymes when modified directly with diazotized *p*-arsanilic acid, derivatives of both α- and δ-chymotrypsin and of chymotrypsinogen A, were prepared under the same standard conditions. The absorption spectra of all three products are closely similar (*vide supra*). Based on the spectra in alkali (Tabachnick and Sobotka, 1960), α- and δ-chymotrypsin contained 0.9 and 0.8 residue of monoazotryosine and 0.1 and 0.08 residue of monoazohistidine per molecule, respectively, values quite similar to those of the arsanilazozymogen (Table I). While the circular dichroic spectra of the two arsanilazochymotrypsins (Figure 6) are very similar to that of the product of activation of arsanilazochymotrypsinogen (Figure 3), they differ distinctly from that of arsanilazochymotrypsinogen (Figure 1). Like the product of activation, both of the arsanilazoenzymes display the *positive* extremum centered near 448 nm, in contrast with the *negative* extremum of the arsanilazozymogen, centered at 428 nm.

The arsanilazochromophore is sensitive not only to changes which occur on zymogen activation but also to those which subsequently affect activity of the resultant enzyme. Addition of the active-site-directed reagent, L-1-tosylamido-2-phenylethyl chloromethyl ketone (2.5 × 10^{-4} M), to either α- or δ-arsanilazochymotrypsin, increases the molecular ellipticity of the Cotton effect at 448 nm 1.7-fold (Figure 6). Other chymotryptic inhibitors, *e.g.*, β-phenylpropionate, *N*-acetyl-L-tryptophan, and *N*-formyl-L-phenylalanine, similarly increase the θ_{448} of the arsanilazochymotrypsins.

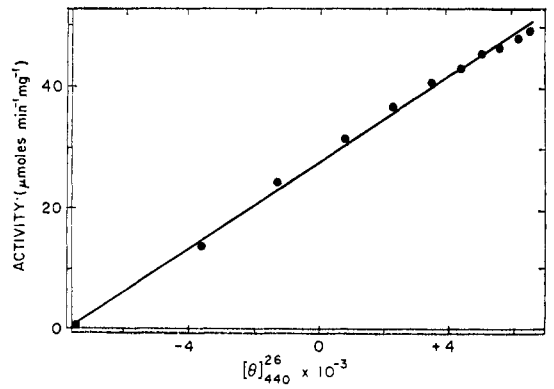

FIGURE 5: Correlation of appearance of *N*-benzoyl-L-tyrosine ethyl esterase activity and change in circular dichroism upon trypsin activation of arsanilazochymotrypsinogen. Activation of the arsanilazozymogen (5 × 10^{-5} M) was carried out in the presence of trypsin (3 × 10^{-7} M) in 0.10 M NaCl–0.05 M Tris buffer, pH 7.5, 26°. Measurements of enzymatic activity were performed as described in Figure 2 and those of changes in circular dichroism as in Figure 5.

Discussion

The conversion of catalytically inactive proteins into enzymes through specific, limited proteolytic cleavage has long been thought to present unusual opportunities for the understanding of the structural basis of enzyme action (Neurath, 1964). These structural changes can involve major degradation of the zymogen molecule, as in the activation of bovine procarboxypeptidase to yield carboxypeptidase A (Brown *et al.*, 1963a,b), or of prothrombin and pepsinogen (Rajagopalan *et al.*, 1966; Magnusson, 1970; Perlmann, 1970).

However, the changes in primary structure can also be relatively minor, as in the activation of bovine trypsinogen (Davie and Neurath, 1955). In bovine chymotrypsinogen A, perhaps the simplest case, activation does not even require the removal of an amino acid residue. The hydrolysis of the Arg-15–Ile-16 peptide bond is sufficient to generate π-chymotrypsin (Jacobsen, 1947; Dreyer and Neurath, 1955; Rovery *et al.*, 1955; Hartley and Kauffman, 1966; Meloun *et al.*, 1966). Autocatalytic cleavage of yet other bonds forms δ-, γ-, or α-chymotrypsins (Dreyer and Neurath, 1955; Desnuelle, 1960; Wright *et al.*, 1968).

In addition, intramolecular rearrangements of tertiary protein structure have been thought important for the proper alignment of groups necessary for catalytic function of the resultant enzymes, though the experimental demonstration has proven difficult. The overall folding of the peptide chain of chymotrypsinogen as determined by X-ray diffraction is similar to that of α-chymotrypsin, and structural changes which accompany formation of the enzyme are small and seemingly subtle (Freer *et al.*, 1970). Studies in solution

employing various methods, including optical rotatory dispersion, circular dichroism, and hydrogen isotope exchange have not been decisive in regard to the existence, nature and extent of conformational changes (*cf.* references cited in introduction). Ambiguities of conclusions reached in different studies may be due, at least in part, to the fact that highly localized changes of the order of fractions of ångstroms may be involved (Freer *et al.*, 1970). These would be at the limits of the resolving power and specificity of such solution methods when applied to native proteins.

Chemical modifications of various proteins with diazonium salts have generated highly specific, extrinsic Cotton effects which seem to reflect local conformations peculiar to and characteristic of the particular proteins examined so far. Since modification can be confined to a single residue, such extrinsic Cotton effects may be capable of signalling local changes with much higher sensitivity than either intrinsic or side-chain Cotton effects (Kagan and Vallee, 1969; Vallee, 1970; Fairclough and Vallee, 1970). The circular dichroic spectra of the arsanilazochymotrypsinogen–chymotrypsin pair seemed of particular interest since the differences in the primary structures of these two proteins are minimal and the extrinsic Cotton effects might provide sensitive signals to measure local conformational changes coincident with the induction of activity.

The chemical modification of chymotrypsinogen A with diazotized *p*-arsanilic acid, when performed under standard conditions at pH 8.5, modifies only a few amino acid residues, and but a total of 1.2 g-atoms of arsenic/mole of protein are incorporated (Table I). The formation of one monoazotyrosyl residue appears to account for the major fraction of reagent bound covalently, as judged both by spectral analysis in alkali and by amino acid analysis. Modification of histidine is negligible. There is no spectral evidence for the formation of bisazolysyl derivatives upon modification at pH 8.5, though amino acid analysis reveals the loss of from 3 to 4 lysyl residues. Following dialysis against 1 mM HCl, the arsenic content can be accounted for largely on the basis of tyrosine modification (Table I). The instability of both triazene and pentazene derivatives of primary amines, particularly in acidic media, could account for such observations (Higgins and Harrington, 1959; Tabachnick and Sobotka, 1959; Smith, 1966). It should be noted that chemical modification of chymotrypsinogen when performed at pH >8.5 is distinctly more extensive but not reflected in the characteristic circular dichroic spectra. Such data were instrumental in selecting pH 8.5 for the present, detailed studies.

Critical roles of tyrosine in the catalytic mechanism of chymotrypsin (Filmer and Koshland, 1964; Labouesse *et al.*, 1964) or of lysyl residues in chymotrypsinogen activation (Chervenka and Wilcox, 1956; Oppenheimer *et al.*, 1966) have not been discerned. Consistent with this, chemical modification of chymotrypsinogen under the present conditions does not interfere with its subsequent activation. Treatment both of the native and modified zymogens with identical amounts of trypsin generates esterase activity at comparable rates (Figure 2). Further, the resultant enzymes exhibit nearly identical K_m and V_{max} values for the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester, and the *intrinsic* Cotton effect appears unaltered by coupling, suggesting that overall tertiary structure is unchanged.[2]

The details of the circular dichroic spectrum of arsanilazochymotrypsinogen between 300 and 600 nm differ from those of other proteins studied thus far (Kagan and Vallee, 1969; Fairclough and Vallee, 1970). The analytical data demonstrating the modification of approximately one tyrosyl residue are entirely consistent with the details of the circular dichroic spectrum both of the arsanilazozymogen and arsanilazo enzyme with extrema at 323 and 428 nm and 321 and 448 nm, respectively (Figures 1 and 3). The assignment of these transitions to an azoaromatic residue, in this case tyrosine, gains strong support from studies of *p*-hydroxyazobenzene (Jaffee *et al.*, 1958), azobenzene (Jaffee and Gardner, 1958), L-*p*-(phenylazo)phenylalanine (Goodman and Kossoy, 1966), poly-L-*p*-(*p'*-hydroxyphenylazo)phenylalanine (Goodman and Benedetti, 1968), poly-L-*p*-(2'-hydroxy-5'-methylphenylazo)phenylalanine (Benedetti and Goodman, 1968), 1,2-dialkyldiazenes (Severn and Kosower, 1969), and of arsanilazotyrosine copolymers (Conway-Jacobs *et al.*, 1970). The rapid activation of arsanilazochymotrypsinogen by trypsin under conditions approximating those known to generate π- and then δ-chymotrypsin progressively inverts the negative extremum at 428 nm which becomes positive with a maximum at 448 nm, with a total change in molecular rotation of 15,000° (Figure 3). The changes in sign position and magnitude are first order with respect to arsanilazochymotrypsinogen concentration and display pseudo-first-order rate constants, directly proportional to the trypsin concentration (Figure 4, Table II).

The close correlation of the time course for the change in the circular dichroic spectrum in the region from 420 to 450 nm with that for the induction of activity would indicate that the circular dichroic changes mirror and amplify conformational changes accompanying zymogen activation, reflecting the kinetics of intramolecular rearrangements (Figure 5). The circular dichroic spectrum of the arsanilazo group, therefore, is a probe of the activation process. Within experimental limits the functional properties of the native and the chemically modified zymogen–enzyme pairs are entirely equivalent, hence the introduction of the probe does not seem to bring about the observed conformational changes.

Conformational rearrangements of arsanilazochymotrypsinogen, subsequent to the induction of enzymatic activity, might reflect in altered asymmetry about residues which need not be directly involved in the active site but which are exposed to chemical modification at the surface of the molecule. It is of interest that only one tyrosine is modified, making the probe characteristics quite selective for a particular region of the molecule, providing studies now in progress prove that the arsanilazo label is confined to a single residue in the primary sequence. A number of the studies employing absorption or difference absorption spectroscopy, optical rotation, or circular dichroism either of the zymogen or its enzymatic products have led to the conjecture that aromatic residues, but particularly tyrosine, contribute to the changes observed (*cf.* references cited). X-Ray diffraction has detailed at least six regions of chymotrypsinogen where local structural changes might relate to activation (Freer *et al.*, 1970). One of these, number V, contains Tyr-146, one of the two tyrosines of chymotrypsinogen A which are

[2] In an earlier study of treatment of α-chymotrypsin with diazotized *p*-sulfanilic acid rapidly inactivated the enzyme (Gundlach *et al.*, 1962). However, this modification was carried out at pH 10.3, higher than that employed here, and extensive coupling not only of tyrosine but also of lysine and of histidine accompanied inactivation.

"exposed" to the solvent.[3] This residue, while not known to be critical either to the process of activation or to the activity of chymotrypsin, has received particular attention. It is involved in the dimerization of α-chymotrypsin and, perhaps, its interconvertibility with γ-chymotrypsin; it is known to be near the active-center region (Gladner and Neurath, 1954; Matthews *et al.*, 1967; Wright *et al.*, 1968; Cohen *et al.*, 1969). Tyrosine-171, while not as close to the active center, is also "exposed" to solvent, and, hence, susceptible to chemical modification. Activation could incur conformational changes involving either or both of these residues, a postulate that can be answered definitively by identification in the primary sequence of the modified residue(s) through studies which are now in progress.

Whatever the chemical details, the optically active arsanilazo probe reflects the conversion of an inactive precursor into its active product, suggesting the occurrence of conformational changes during activation. In 6 M guanidine hydrochloride the absorption spectra of azoproteins are virtually unaltered while their extrinsic Cotton effects are abolished (Kagan and Vallee, 1969; Fairclough and Vallee, 1970). Such sensitivity to denaturing conditions indicates responsiveness of side-chain azochromophores to the distinctive environmental features of specific protein conformations, such as seem to exist even in closely related, structurally similar proteins as those here studied. Similarly, L-1-tosylamido-2-phenylethyl chloromethyl ketone can affect the circular dichroic spectrum of the enzyme, once generated (Figure 6). X-Ray diffraction studies of such derivatives might relate these circular dichroic data, thought to reflect highly specific and localized conformational changes, to the particular region(s) where local structural changes might result from activation, as judged by crystallographic studies (Freer *et al.*, 1970).

At present, definitive assignments of the origin of optical activity in complex systems would seem tenuous (Schellman and Schellman, 1964; Schellman, 1968), though the requisite procedures and calculations have been summarized and detailed (Tinoco and Cantor, 1970), and performed, *e.g.*, for myoglobin (Hsu and Woody, 1969), among others.

The effects of solvents, ionic strength, and pH of the circular dichroic spectra of various diazotized poly-L-phenylalanine derivatives form a basis for such calculations on azoproteins. Possible mechanisms for the inversion of the sign of the Cotton effect in poly-L-*p*-(2′-hydroxy-5′-methylphenylazo)-phenylalanine when dissolved in dimethylacetamide and hexafluoro-2-propanol, respectively, have been discussed (Benedetti *et al.*, 1968; Benedetti and Goodman, 1968; Goodman and Benedetti, 1968). This model, among those known, pertains perhaps most directly to the present system. The establishment of a definitive mechanism encounters problems even for such a relatively well defined system. Extrapolation of the data to the present case suggests that vicinal alterations could bring about changes in sign, but other alternatives cannot be excluded without further experimental work. However, such studies on models indicate what direction future efforts might take to discern the mechanism of the inversion in the activation of arsanilazochymotrypsinogen. Operationally, the present data provide an instrumentally magnified readout of a presumed change in conformation whose elucidation may contribute importantly to the understanding of the mechanism of zymogen activation.

[3] The six segments of the backbone chain which exhibit relatively large differences between the zymogen and enzyme are composed of from two to seven residues. As a basis for possible conformational differences, distances between α-carbon atoms of identical residues in the two molecules were compared, and only the 21 residues whose α-carbon atoms moved more than 3.6 Å subsequent to activation were considered. Segment V was the only one of these which contained a tyrosyl residue, *i.e.*, Tyr-146. The displacement of its α-carbon atom was 4.6 Å. However, 19 of the other 20 residues were displaced by the same or greater distances, *i.e.*, up to 11.3 Å for Ile-16 (Freer *et al.*, 1970).

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2-Phenylethaneboronic Acid, a Possible Transition-State Analog for Chymotrypsin*

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ABSTRACT: 2-Phenylethaneboronic acid, hydrocinnamamide, and 2-phenylethanesulfonate are competitive inhibitors of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate. The dissociation constants (K_i) of the enzyme–inhibitor complexes have been determined as a function of pH over the pH range between 5 and 10. 2-Phenylethaneboronic acid binds most tightly to the form of chymotrypsin in which the imidazole group of histidine-57 at the active site exists as the free base (K_i = 0.04 mM at pH 8), whereas the state of protonation of this imidazole does not significantly affect the binding of hydrocinnamamide (K_i = about 6 mM between pH 5 and 8).

2-Phenylethanesulfonate binds more tightly to the form of the enzyme in which the imidazole is protonated (K_i = 2.9 mM at pH 5) than it does to the form in which the imidazole is unprotonated (K_i = 30 mM at pH 8). These facts are used in conjunction with the chemistry of boronic acids and the present knowledge of the structure and mechanism of action of chymotrypsin to deduce that one possible structure for the complex between chymotrypsin and the boronic acid is the structure in which the hydroxyl group of serine-195 has added to boron to form a negatively charged tetrahedral adduct and in which the imidazole is protonated, $^{+}HN\text{–}E\text{–}CH_2\text{–}O\text{–}B(R)\cdot(OH)_2^{-}$. Such a structure is an approximate analog of the transition states for acylation and deacylation of serine-195 which occur during catalysis.

A transition-state analog for an enzyme is a compound which forms a stable complex with the enzyme in which complex the structure of the analog resembles the substrate portion of the transition state (structure of highest energy) for the enzymatic conversion of bound substrate into bound product (Pauling, 1948; Wolfenden, 1969). The crystal structure of an

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