Iodination of a Single Tyrosine in Crystals of α-Chymotrypsin*

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ABSTRACT: Crystals of α-chymotrypsin were reacted with I_3^- at pH 4 and 5. Crystallographic studies showed irreversible substitution at two sites per molecule which were compatible with diiodination of a single tyrosine residue. Electrophoresis of a complete enzymatic digest of the iodinated [^{131}I]crystals revealed monoiodotyrosine and diiodotyrosine as the only reaction products. The change in the ratio of diiodotyrosine to monoiodotyrosine during the course of the reaction implied that the reaction was completed by the diiodination of a single tyrosine residue.

Amino acid analysis of the radioactive peptides obtained from a tryptic fingerprint indicated conclusively that the iodinated residue was tyrosine-171. Tyrosine-146, which solution studies had previously shown was the most exposed tyrosine residue, was not iodinated. The difference between the pattern of iodination in solution and in the crystals is explained by molecular interactions revealed in the crystal structure determination of tosyl-α-chymotrypsin (Matthews *et al.* (1967), *Nature 214*, 652). Although this derivative was not required to provide phases in the structure determination of α-chymotrypsin, it did contribute to the interpretation of the electron density map and the correlation of the electron density maps derived from the monoclinic and tetragonal crystal forms.

In this paper experiments will be described in which a single residue of α-chymotrypsin, tyrosine-171, was iodinated by reacting crystals of α-chymotrypsin with I_3^-. The iodine atoms were located in the crystal structure by difference Fourier projections at 3.8-Å resolution; and the type of amino acid as well as its location in the sequence was determined by using radioactive $^{131}I_3^-$.

The preparation of this crystalline iodinated derivative was undertaken for four reasons, all of which were related to the crystallographic structure determination of tosyl-α-chymotrypsin at atomic resolution (Matthews *et al.*, 1967). The first reason and principal motivation for this study was to determine the position of a specific residue of the sequence in the crystal structure and thereby provide a guide point for the interpretation of the electron density map. The second purpose of this study can be considered an extension to the solid state of the conventional "surface probe experiment" wherein the "surface" of a protein molecule is defined in terms of the reactivity of the protein's functional groups with an ambient reagent. The third reason for preparing a derivative such as the one described here was to provide a convenient method for relating polymorphous crystal forms of the same or homologous protein molecules. By covalently bonding a sufficient number of heavy-atom markers to specific positions in the protein molecule it was felt that it should be possible—at least in certain cases—to use low-resolution projection studies to relate the orientation of a molecule in one crystal lattice to the orientation of the same (or a similar) molecule in a different crystal lattice (Perutz *et al.*, 1964; Matthews *et al.*, 1968). The advantage of using covalently bonded heavy-atom markers is the assurance which can be gained through chemical methods that the markers in the various crystal forms are linked to the same residue. The fourth reason for preparing this derivative was to produce a heavy-atom isomorphous derivative for the purpose of phase determination in the crystallographic structure determination.

For each of these purposes it was desired that the derivative be highly substituted at a limited number of sites. Moreover, for the purpose of providing a specific residue marker a heavy atom was required which could be isotopically labeled and covalently bonded to the structure. Iodination presented a promising approach since in the case of chymotrypsin the preferential (but not exclusive) iodination of tyrosine-146 had been independently reported by Glazer and Sanger (1963) and by Dube *et al.* (1964a,b). Attempts to crystallize the protein which had been iodinated in solution failed (B. A. Jefferey, private communication); however, the selectivity and simplicity of the reaction suggested that a similar reaction should be attempted in the crystal.

Iodinating reagents such as I_3^-, ICl, or OI^- (all ultimately yielding I^+) have been extensively used with enzyme solutions to probe the accessibility of tyrosine and histidine side chains and as a means of selectively modifying these amino acids (*e.g.*, Covelli and Wolff, 1966; Kenkare and Richards, 1966; DiSabato, 1965; Pressman and Reholt, 1961). Although side reactions such as oxidation of tryptophan have been reported (Wolff and Covelli, 1966; Hartdegen and Rupley, 1967), the principal effect of these reagents on proteins is to iodinate the tyrosine and histidine side chains.

Iodination of *crystalline* proteins has been attempted independently by several groups. Venkatappa and Steinrauf (1967) iodinated a histidine residue of crystalline egg-white lysozyme (triclinic and monoclinic) and identified the product chemically but not crystallographically. Kretsinger (1968) crystallized myoglobin in the presence of I_3^- and observed peaks in the difference Fourier synthesis in the region of two tyrosine side chains. Wykoff *et al.* (1967) used an iodinated

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tyrosine of ribonuclease S as a marker to help relate the electron density map of ribonuclease S which is in a trigonal crystal lattice to that of ribonuclease A which is in a monoclinic crystal form (Kartha *et al.*, 1967). Most germaine to this paper is the recent study of Matthews *et al.* (1968) who have used an iodinated tyrosine residue of γ-chymotrypsin as one of several markers to establish the coordinate transformation that relates the 5.5-Å map of tetragonal γ-chymotrypsin to the 2.0-Å resolution map of monoclinic α-chymotrypsin (Matthews *et al.*, 1967).

Experimental Section

Materials and Methods

I_3^- Stock Solution. Sublimed crystals of I_2 (BDH) were used to prepare a 0.100 M (25.4 mg/ml) solution of I_2 in methanol. Two millimoles (332 mg) of KI (BDH, Analar) was dissolved in a minimal amount of water and 20.0 ml of the I_2 solution was added with stirring. The mixture was diluted with water to 100 ml with precipitation of a very small amount of what appeared to be I_2 crystals. This "I_3^- stock solution" was sealed in an amber bottle and stored in the dark. In order to determine the molar specific radioactivity of the $^{131}I_3^-$ preparation, the concentration of total soluble iodine was assayed by thiosulfate titration following oxidation of the I^- component by nitrite (Koltoff and Belcher, 1957). The stock solution was found to be 0.0176 (±1.0%) M in I_3^-.

Iodination Reaction. Crystals of α-chymotrypsin grown as described by Sigler *et al.* (1966) were transferred to 65% saturated $(NH_4)_2SO_4$–0.06 M citrate (sodium) (pH 4.0) or 70% saturated $(NH_4)_2SO_4$–0.06 M succinate (sodium) (pH 5.0–5.2) and allowed to equilibrate for at least 24 hr. Five milliliters of fresh supernatant (either pH 4.0 or 5.0) was added to each vial containing about 10 mg (~0.4 μmole) of crystalline enzyme. One-tenth milliliter (1.8 μmoles) of the "stock I_3^-" solution was added directly to each vial, rapidly mixed, and the vial was tightly sealed to prevent the escape of I_2 vapor which results from the disproportionation of I_3^- in solutions of high salt concentration. After 8 days at room temperature in the dark, the reaction was terminated by rinsing the amber-colored crystals with a solution which was 65% saturated with $(NH_4)_2SO_4$, 10^{-4} M in KI, buffered at pH 4.0 with 0.06 M citrate (sodium), then allowing them to equilibrate for an additional 48 hr with this I_2-free mother liquor. During this time the unreacted triiodide diffused out and the crystals became colorless. The supernatant solution was replaced twice more at 24 hr intervals with a similar $(NH_4)_2SO_4$ solution which contained no I^-, and 6 days after the termination of the reaction the water-clear, perfectly intact crystals were mounted in capillaries for X-ray study.

Radioactive Iodination. Crystals of α-chymotrypsin were iodinated in the manner described above for the X-ray diffraction studies, except that $^{131}I^-$ was added to the stock I_3^- solution and much larger batches of crystals were used. In order to study the course of the iodination the concentration of I_3^- and the duration of the iodination were varied in some experiments (Table II).

In a typical experiment 9.0 ml of the stock I_3^- solution was added to 2.0 ml of 0.2 M citrate (sodium) buffer (pH 4.0). The citrate buffer I_3^- stock solution (7 ml) was added to 0.05 ml (1 mC) of alkaline, carrier-free, and reducer-free $^{131}I^-$ (Radiochemical Centre, Amersham) to give a 0.014 M stock solution of $^{131}I_3^-$. The $^{131}I_3^-$ stock solution (1.5 ml, 21 μmoles) was added to 35 ml of mother liquor (either pH 4.0 or 5.0) in equilibrium with about 160 mg (6.4 μmoles) of crystalline α-chymotrypsin.

Crystallographic Characterization of the Reaction Product. Precession photographs (17°, 2.8 Å) and (12°, 3.8 Å) were taken of the axial zones. Difference Fourier syntheses of the principal projections (Figure 1) were calculated using the coefficients $m(|F_{Iod}| - |F_{Nat}|)\exp i\phi$, where $|F_{Iod}|$ and $|F_{Nat}|$ are the appropriately scaled observed structure amplitudes of the iodinated (Iod) and native (Nat) proteins, respectively; ϕ is the centroid phase angle, and m is the figure of merit used in the calculation of the structure of tosyl-α-chymotrypsin (Matthews *et al.*, 1967).

Chemical Characterization of the Reaction Products. Since iodinated tyrosine is unstable under conditions of acid hydrolysis of peptides (Glazer and Sanger, 1963) the degradation of the iodinated protein to its constituent amino acids was carried out enzymatically as follows: 8 mg of [^{131}I]-α-chymotrypsin[1] was digested by 2 mg of pepsin (Worthington) for 8 hr at 37° in 1 ml of 5% formic acid. After the solution was brought to pH 8 by the addition of 2 M triethylammonium bicarbonate, approximately 1 mg of trypsin (Worthington), subtilisin (Novo), and pronase of *Streptococcus griseus* (gift of Dr. B. S. Hartley) were added successively at 4-hr intervals to the mixture at room temperature. Four hours after the addition of pronase the faintly opalescent hydrolysate was filtered (no radioactivity in the precipitate), diluted to 20 ml with water, and lyophilized. The residue was redissolved and relyophilized several times to remove the volatile electrolytes.

The digest was "spotted" on Whatman No. 3MM at a density of 0.03 μmole of α-chymotrypsin/cm and subjected to a 6-kV ionophoresis at pH 2.1 (8% acetic–2% formic acid). The position of monoiodotyrosine and diiodotyrosine on the ionogram were indicated by authentic samples (Koch-Light, checked by melting point) spotted at 0.05 μmole/cm. Following radioautography the ionograms were stained with collidine–ninhydrin to locate the position of the iodotyrosine markers and other amino acids used as standards.

In order to determine the position of the radioactive amino acid in the sequence it was necessary to separate the iodinated enzyme into its component chains, split the radioactive chain into tryptic peptides, and identify the radioactive peptides by their amino acid composition. Crystalline [^{131}I]-α-chymotrypsin was completely reduced, alkylated, and separated by ion-exchange chromatography as described by Hartley (1964). In a typical experiment crystals were dissolved in a minimum amount of 0.02 M acetic acid, desalted on a G-25 Sephadex column, equilibrated with 0.01 M acetic acid, and lyophilized. The lyophilized protein (30 mg, 1.3 μmoles) was dissolved in 2 ml of 0.02 M acetic acid (the low pH serving to prevent autolysis) to which was added 1.7 g of crystalline urea. Two milliliters of 10 M urea, buffered with 0.5 M Tris-acetate, was added and the pH was adjusted to 8.6 with Tris (free base). The solution was deaerated, treated with 30 μl (400 μmoles) of redistilled 2-mercaptoethanol (Koch-

[1] [^{131}I]-α-Chymotrypsin designates α-chymotrypsin iodinated with ^{131}I without specifying the extent of iodination.

TABLE I: Unrefined Fractional Coordinates of the Iodine Atoms Derived from the Difference Fourier Projections Shown in Figure 1.[a]

Binding Site	Projection	Resolution (Å)	Molecule a			Molecule b		
			x	*y*	*z*	*x*	*y*	*z*
I_1	(*h01*)	3.8	0.32 (0.68)		0.57 (0.43)	0.32 (0.68)		0.28 (0.72)
	(*hk0*)	3.8	0.32	0.63		0.32	0.37⁻	
	(*0kl*)	3.8		0.63	0.56		0.38	0.28
			0.32	0.63	0.56	0.32	0.37	0.28
I_2	(*h01*)	3.8	0.33 (0.67)		0.48 (0.52)	0.33 (0.67)		0.37 (0.63)
	(*hk0*)	3.8	0.33	0.63		0.33	0.37	
	(*0kl*)	3.8		0.63	0.48		0.38	0.37⁻
			0.33	0.63	0.48	0.33	0.37	0.37

[a] Coordinates in parentheses for the (*h01*) projection are those represented by targets in Figure 1 and are related to their companion values by crystallographic symmetry. Molecules a and b are related by local dyad symmetry.

Light), and incubated for 4 hr at 37°. The excess mercaptoethanol was removed by passing the reaction mixture through a G-25 Sephadex column previously equilibrated with deaerated and deionized 8 M urea–0.1 M Tris-acetate (pH 8.6)–0.001 M dithiothreitol (Calbiochem) and collected in a 10-ml volume. Fifty microliters of 1.0 M ammonium iodoacetate (50 μmoles), pH 8.6, was added to the reduced protein solution. The alkylation was terminated after 30 min by the addition of 250 μl of 2-mercaptoethanol.

After the reduced and carboxymethylated chains were dialyzed against 8 M urea–0.02 M Tris-acetate (pH 8.0), they were separated on a 2 × 35 cm DEAE (Whatman DE-52) column at pH 8.0 using a 10-ml sample and a 240-ml salt gradient extending from 0.02 to 0.50 M Tris-acetate (Figure 5).

The protein concentration of the effluent was monitored by the absorbance at 282 mμ. Since the B and C chains each contain four tryptophan residues (Hartley and Kauffman, 1966) the molar extinction coefficient for each chain can be considered approximately one-half that of the total protein, that is, 2.5×10^4 at 282 mμ (Schwert and Kaufman, 1951). The A chain has no significant chromophores Hartley and Kauffman (1966) and because of its small size (13 residues) it is lost in the gel filtration that follows the reduction step.

Results

The Position of the Iodine Atoms in the Crystal Structure. The change in electron density produced by iodinating α-chymotrypsin in the crystalline state at pH 5.0 (±0.1) is shown in Figure 1. These difference Fourier projections show two pairs of heavy atoms in the crystallographic asymmetric unit at the positions listed in Table I. Each pair of heavy atoms represents the iodination of a single molecule by two iodine atoms since the pairs are exactly related by the same local twofold axis which is known to relate the two molecules of the asymmetric unit (Blow *et al.*, 1964; Matthews *et al.*, 1967). The distance between the atoms in each pair is 6.0 Å which agrees with the value of 6.05 Å observed by Hamilton and Steinrauf (1967) for the interiodine distance in diiodotyrosine. The reaction appears strikingly specific with a minimum of density shifts in the region of the replacement. It is perhaps noteworthy that the electron density at site I_2^b is noticeably lower than at the other sites both in the (*h0l*) and (*0kl*) projection. The marginally depressed occupancy of the I_2^b position is unequivical when the overall level of substitution is diminished by carrying out the reaction at pH 4.0. Presumably this kinetic asymmetry reflects a small difference in the environments of the dyad-related tyrosines, which this author could not appreciate by simply inspecting the unaveraged map of α-chymotrypsin (Matthews *et al.*, 1967). Thus the asymmetry of the iodination rates appears to amplify an otherwise undetectable departure from the local twofold symmetry.

The Chemical Characterization of the Reaction Product. IODINATED SPECIES. The conclusion tentatively drawn from the crystallographic results, that the iodinated residue was tyrosine, was confirmed by high-voltage ionophoresis of a complete enzymatic digest of the reaction product. Figure 2 is a radioautograph which shows two (and only two) distinct, slow-moving bands of radioactivity which have exactly the same mobility as authentic standards of monoiodotyrosine and diiodotyrosine. No further characterization was felt to be necessary in view of the crystallographic evidence.

STOICHIOMETRY. The number of atoms of iodine incorporated per molecule of chymotrypsin was established by comparing the molar specific radioactivity of the iodinating solution with that of the iodinated protein. From Table II it can be seen that up to 1.67 atoms of iodine was incorporated per molecule of α-chymotrypsin and that the extent of the reaction is a function of the concentration of I_3^-, the duration of the exposure and the pH. However, it is the relative distribution of iodine between monoiodotyrosine and diiodotyrosine which is of principal interest. This was determined by measuring the radioactivity of the bands produced on high-voltage ionophoresis of the complete enzymatic digest of each of the preparations listed in Table II (Figure 3). From the number of iodine atoms per molecule and their relative distribution between mono- and diiodotyrosine

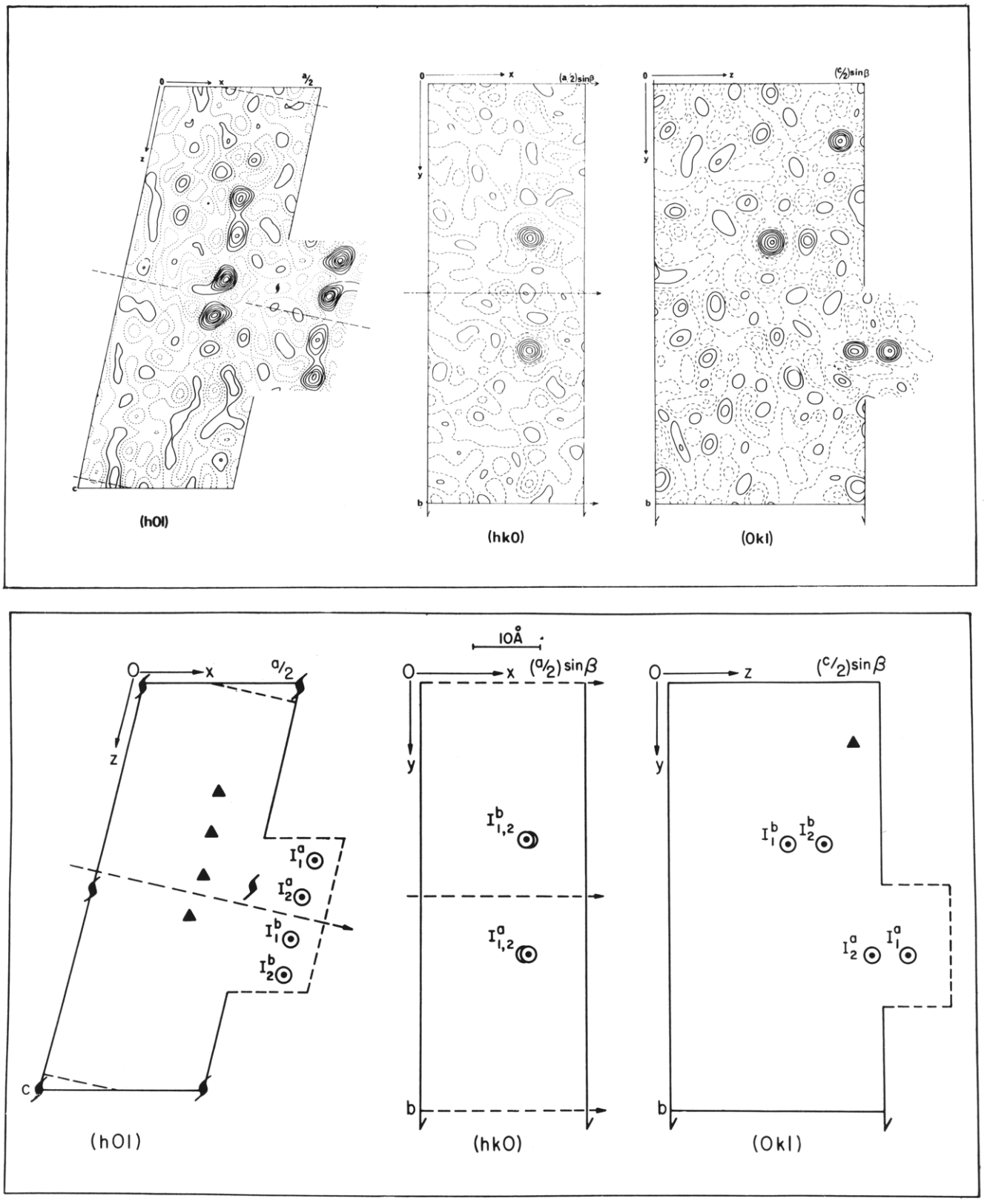

FIGURE 1: (a) Difference Fourier projections at 3.8-Å resolution comparing α-chymotrypsin iodinated in the crystalline state at pH 5.0 with the native enzyme. (*h*0*l*) and (0*kl*) calculated with phases and figures of merit for the native enzyme derived from the $PtCl_4^{2-}$ and pipsyl derivatives (Sigler *et al.*, 1966). (*hk*0) calculated with phases used to compute the Fourier synthesis of tosyl-α-chymotrypsin (Matthews *et al.*, 1967). See text for coefficients. (b) Schematic representation of the distribution of iodine sites. Targets indicate positions of iodine atoms which are in the domain of the local twofold axes shown by the broken lines. (▲) Sites related to the targets by the symmetry of the space group.

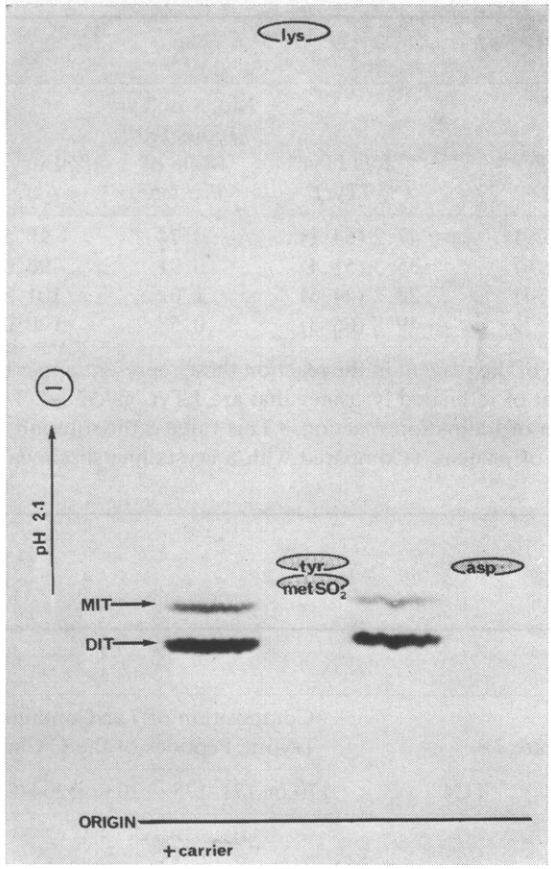

FIGURE 2: Radioautograph of a pH 2.1 ionogram of the complete enzymatic digest of α-chymotrypsin crystals iodinated with $^{131}I_3^-$. The positions of diiodotyrosine (DIT) and monoiodotyrosine (MIT) were determined by authentic standards stained with collidine–ninhydrin. The extent of ionophoresis is indicated by the positions of other amino acids included in the experiment as standards.

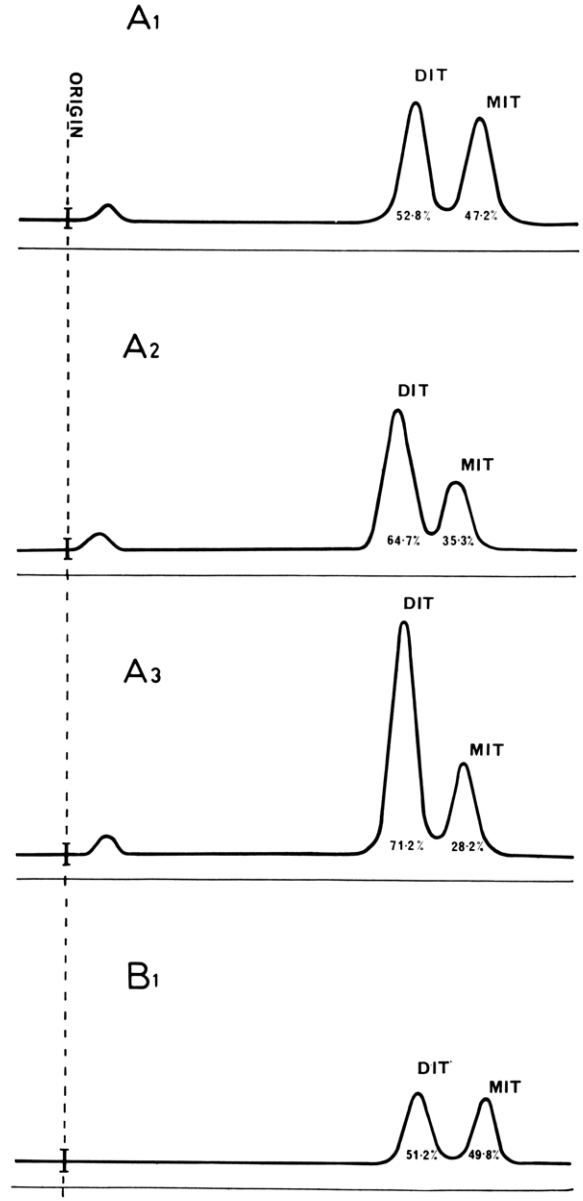

FIGURE 3: The distribution of radioactivity between diiodotyrosine and monoiodotryosine as obtained by scanning ionograms similar to the one shown in Figure 2 and confirmed by scintillation counting of the excised radioactive bands. The letters refer to the specimen designation in Table II. The percentage represents the fraction of the total radioactivity present in the peak. The trace of cationic radioactivity consistently observed near the origin was not identified. It is not thyroxin or triiodothyronine.

the number of tyrosine residues per molecule which react with iodine can be deduced (Table II). A plot of the number of tyrosine residues which are iodinated as a function of the extent of iodination reveals that as the extent of iodination approaches two iodine atoms per molecule, the number of tyrosine residues involved in the reaction approaches one (Figure 4). This is consistent with the X-ray findings and the analysis of the iodinated peptides (see below) both of which indicate that diiodination of a single tyrosine residue is the result of prolonged exposure of α-chymotrypsin crystals to I_3^-.

The Location of the Iodinated Tyrosine in the Sequence. There are four tyrosines in α-chymotrypsin; two in the B chain and two in the C chain (Hartley and Kauffman, 1966). The protein was therefore reduced and carboxymethylated; and the B and C chains were separated by ion-exchange chromatography, as described by Hartley (1964) (see Materials and Methods). It can be seen from Figure 5 that almost all of the radioactive iodine is associated with the C chain, the identity of which was confirmed by amino acid analysis (Figure 6). The possibility that the radioactivity which eluted with the C chain was a small autolytic fragment running with excluded volume, was ruled out by the fact that the radioactivity and optical density were eluted as a single peak in 8 M urea on G-50 Sephadex.

The question of which tyrosine of the C chain was iodinated was solved by characterizing radioactive tryptic peptides on the basis of their amino acid composition. A trypsin digest of the reduced and carboxymethylated protein as well as the isolated C chain produced an appreciable precipitate which was removed by filtration and found to contain less than 16% of the total radioactivity. The radioactive soluble peptides were located on fingerprints by radioautography (Figure 7). Except for a trace of radioactivity in the neutral band, all of the radioactive spots in the whole protein could be attributed to peptides from the C chain. Weak

TABLE II: Summary of the Iodination of α-Chymotrypsin Crystal with $^{131}I_3^-$.

Sample	pH	$[I_3^-]$[a] (mM)	Time (days)[c]	gram-atoms of I/Mole of Protein	% I as I_2Tyr (% I_2Tyr)[b]	% I as ITyr (% ITyr)	Moles of Tyr[d] Iodinated/ Mole of Protein	% Enzyme Act.[e]
A_1	5.0	0.3	8	1.00	52.8 (35.9)	47.2 (64.1)	0.74	90.5
A_2	5.0	0.6	8	1.36	64.7 (48.6)	35.3 (51.4)	0.91	96.0
A_3	5.0	0.6	30	1.67	71.2 (55.4)	28.2 (44.6)	1.07	101.5
B_1	4.0	0.6	8	0.77	51.2 (33.7)	49.8 (66.3)	0.58	100.0

[a] Starting concentration: due to leakage of I_2 vapor and consumption of the reagent in the reaction these represent upper limits and poor approximations of the actual values. [b] % I_2Tyr = the per cent of iodinated tyrosines that are I_2Tyr, given by $I_2Tyr = [(100)(\%\ I\ as\ I_2Tyr)/2]/[(\%\ I\ as\ I_2Tyr/2) + (\%\ as\ I\ ITyr)]$. [c] Duration of iodination reaction. [d] This value is the solution, x, to the equation: $2(\%\ I_2Tyr)x + (\%\ ITyr)x = 100$ gram-atoms of I/mole of protein. [e] Compared with a crystalline specimen subjected to the same purification scheme.

TABLE III

Composition of [^{131}I]Tryptic Peptides Eluted from Ionogram (Figure 7)[a]						Composition of Tyr-Containing Tryptic Peptides of the C Chain[b]	
	TC1[c]	TU1	TC2	TC3	TC4	170 or 171–175	203 or 204–230
Ala	−	−	−	−	−	0	2
Arg	−	−	−	−	−	0	1
Asx	−	−	−	−	−	0	1
Cys	−	−	−	−	−	0	1
Gly	+	+	+	+	+	1	4
Ile	−	−	−	−	−	0	1
Leu	−	−	−	−	−	0	1
Lys	+	+	+	+	+	2 or 1	1 or 0
Pro	−	−	−	−	−	0	1
Ser[d]	−	−	−	−	−	0	5
Thr	+	+	+	+	+	1	4
Val	−	−	−	−	−	0	3

[a] The reason for four rather than one tryptic peptide is explained in the text. [b] Since both regions start with the sequence Lys-Lys each can give rise to two possible tryptic peptides as shown in Figure 8. [c] (+) indicates a definite peak and (−) means the unequivocal absence of a peak on the amino acid analysis of the acid-hydrolyzed peptides. [d] In all the peptides a very small peak was noted at the position corresponding to serine but because a similar peak was observed in the eluate from any region of the paper, it was considered a contaminant.

ninhydrin stains revealed no overlapping of the radioactive spots by neighboring nonradioactive peptides. The amino acid composition of the radioactive peptides is shown in Table III. The presence of only lysine, threonine, and glycine, and the notable absence of amino acids such as arginine, valine and alanine, clearly established that *tyrosine-171*, rather than tyrosine-228, was iodinated (Figure 8). The presence in the fingerprint of four rather than one radioactive tryptic peptides is explained by the doubling which would result from incomplete cleavage of the Lys–Lys bonds in the tryptic digestion. Further doubling is presumed to result from the difference between the pK_a of the phenolic hydroxyl of monoiodotyrosine (pK_a = 8.5) and that of diiodotyrosine (pK_a = 6.5).

Discussion

Role of Iodotyrosine-171 in the Interpretation of the High-Resolution Electron Density Map of α-Chymotrypsin. Once a map such as that of tosyl-α-chymotrypsin has been interpreted it is difficult to decide in retrospect the relative value of any particular bit of ancillary information. Clearly fixing the position in the crystal structure of one or two residues of the sequence is not sufficient to allow one to interpret

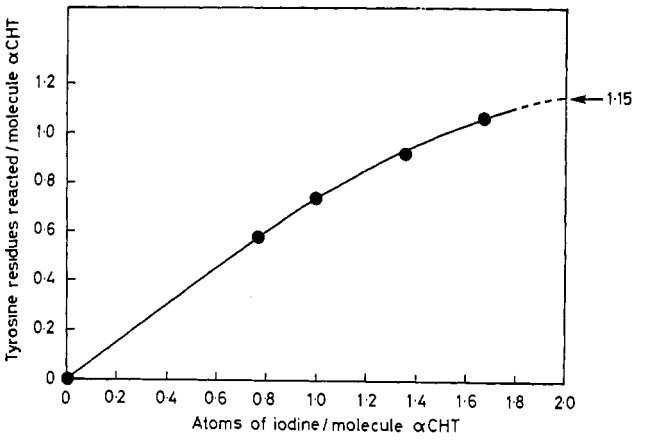

FIGURE 4: The number of iodinated tyrosine residues per molecule of α-chymotrypsin (column 8, Table II) plotted as a function of the extent of iodination in the crystal (column 5, Table II). Curve extrapolated to two iodine atoms per molecule of α-chymotrypsin.

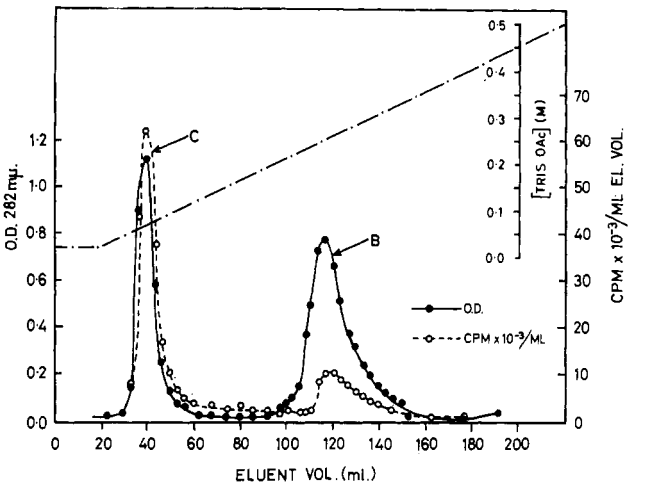

FIGURE 5: Chromatographic separation of reduced and carboxymethylated B and C chains of α-chymotrypsin iodinated in the crystalline state with $^{131}I_3^-$. DEAE-cellulose column, pH 8.0, gradient is 0.02–0.50 M Tris-acetate (see text).

an otherwise unintelligible map; indeed, it is probable that electron density map of tosyl-α-chymotrypsin would have been correctly interpreted without labeling either tyrosine-171 or serine-195 (Sigler *et al.*, 1966). On the other hand, there is no question that by imposing stringent constraints on the interpretation these markers or guide points were a considerable aid in the structure analysis. Because of the certainty with which the positions of the labeled residues were known these guide points, in effect, served as "brass tacks" in the model building process.

Role of I_3^- as a Surface Probe for Crystalline α-Chymotrypsin. It is probably more meaningful to discuss the "surface" of a protein in an operational rather than a strictly topological sense. Thus the "surface" of a protein is simply that set of residues that react with one or more molecular probes, at or above a specified minimum rate. In this context the surface of a protein in the crystal differs from that in solution in two ways. First, the crystal structure produces exactly the same environment for each molecule (or, in this case, pair of molecules) in contrast to the spherically averaged condition of macromolecules in solution. Moreover, in the crystal there is likely to be a much sharper distribution of conformations (virtually a "line" spectrum) than in solution. Thus the "surface" of a molecule will almost certainly appear more well defined in the crystal than in solution. Second, the reactivity of the residues which comprise the surface of the protein in the crystal will be affected by steric restrictions imposed by the interactions between adjacent molecules which stabilize the lattice.

Both of these effects are well illustrated by the differences in the patterns of iodination of dissolved and crystalline α-chymotrypsin. In solution the rate of iodination of tyrosine-146 is initially three times that of the rate for the next most reactive residue (Dube *et al.*, 1964b). However, the preferential reactivity of tyrosine-146 becomes less distinct in solution as the reaction proceeds, to the extent that substantial iodination at several subsidiary sites was observed at a point in the reaction when less than half of the tyrosine-146 residues had reacted (Dube *et al.*, 1964b). In contrast, when the reaction was carried out in crystals of the α form, tyrosine-171 could be 84% diiodinated (Table II) while producing only a trace of subsidiary reaction products (Figure 3).

Even more striking is the restrictive influence of the crystal packing interactions. In solution tyrosine-146 is not only the most rapidly iodinated residue (Glazer and Sanger,

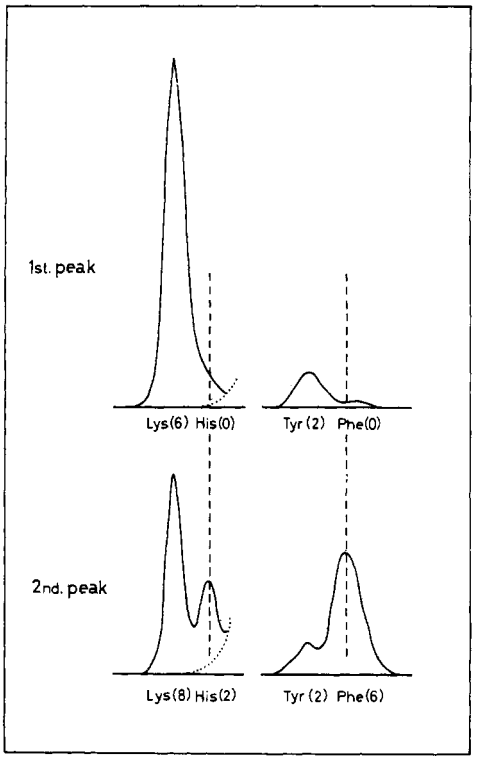

FIGURE 6: Identification of the chains separated by DEAE-cellulose chromatography as shown in Figure 5. The C chain can be identified as the first peak from its amino acid composition, since it has no histidine nor phenylalanine (Hartley and Kauffman, 1966).

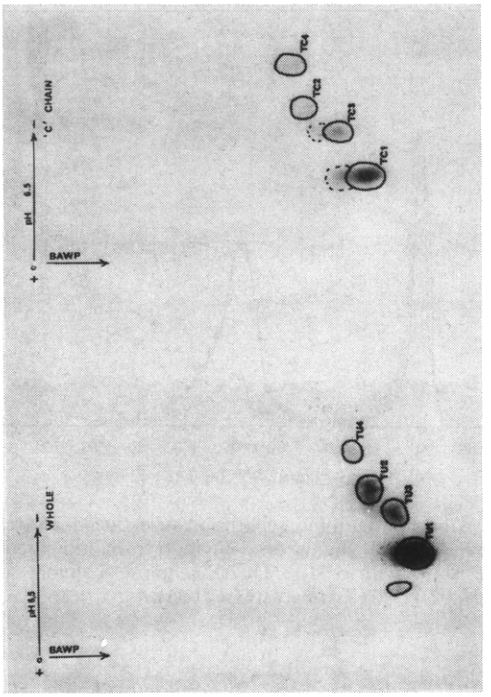

FIGURE 7: Radioautographs of "fingerprints" of tryptic digests of α-chymotrypsin iodinated in the crystalline state with $^{131}I_3^-$. Ionophoresis in pyridine formate (pH 6.5) was followed by descending chromatography in butanol, acetic acid, water, and pyridine. (a) Digest of the entire protein and (b) digest of isolated C chain (Figure 5). Except for a trace of radioactivity in the neutral band, the pattern obtained from the whole protein is identical with that obtained from the isolated C chain.

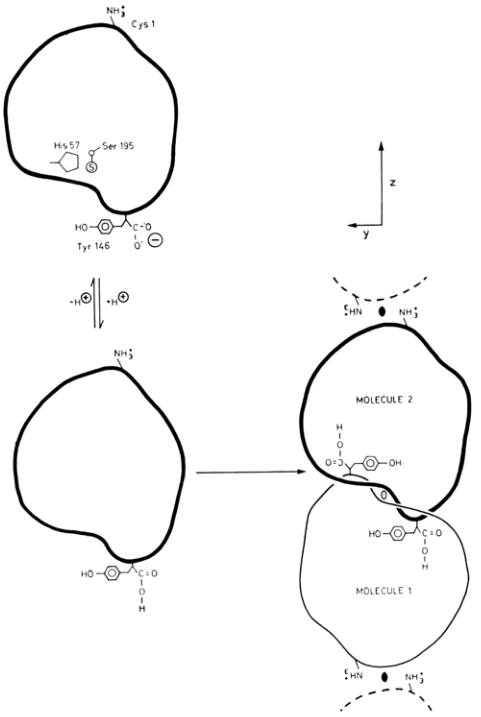

FIGURE 9: Schematic representation, viewed in projection down the noncrystallographic dyad, of the dimerization interaction between molecules of α-chymotrypsin which accounts for the inability to iodinate tyrosine-146 in the crystal. This scheme suggests that the formation of the local dyad axis which characterized the α lattice, and probably the formation of dimers in solution requires the presence of an unchanged carboxyl-terminal tyrosine-146. This molecular interaction is discussed in detail by Sigler *et al.* (1968).

1964; Dube *et al.*, 1964b) but it is readily removed from native α-chymotrypsin by carboxypeptidase (Gladner and Neurath, 1954). Despite this conclusive evidence for the presence of tyrosine-146 on the surface of the molecule it was completely unreactive in the crystals of α-chymotrypsin under conditions where tyrosine-171 is 84% diiodinated (Table II and Figures 5 and 7). The reason why tyrosine-146 in the crystal form does not react with I_3^- was immediately apparent from an analysis of the molecular interactions in the electron density map of tosyl-α-chymotrypsin (Sigler *et al.*, 1968). Tyrosine-146 does protrude well out from the bulk of the molecule as the solution studies had suggested. However, in the packing of the molecules around one of the local dyads which characterizes the α crystal form, the phenolic side chain of tyrosine-146 is embedded in the dyad-related neighbor in a way that would make it inaccessible to iodinating reagents. This is shown schematically in Figure 9. This explanation is supported by the fact that Cohen *et al.* (1969) have iodinated tyrosine-146 as well as tyrosine-171 in γ-chymotrypsin, the tetragonal crystal form of the same molecule, in which the arrangement of the molecules would indicate that tyrosine-146 is not involved in intermolecule contacts (Matthews *et al.*, 1968). Indeed it has been suggested (Sigler, 1967; Wright *et al.*, 1968) that the very existence of the α-crystal form is due to stabilizing molecular interactions involving the protonated form of the carboxyl terminal 146.

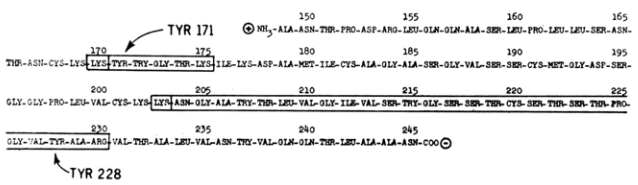

FIGURE 8: The amino acid sequence of the C chain (Hartley and Kauffman, 1966). The outlined portions of the sequence represent the two tyrosine-containing tryptic peptides. Note: both begin with Lys–Lys; therefore each region will produce a pair of tryptic peptides.

The Use of Heavy Atoms to Relate the Orientation of a Molecule in One Crystal Lattice to That of a Similar or Homologous Molecule in a Different Crystal Lattice. In the study by Matthews *et al.* (1968) cited in the introduction, five markers were used to deduce and refine the coordinate transformation relating the orientation of the 2-Å map of the chymotrypsin molecule in the α or monoclinic lattice to that of the 5.5-Å map in the γ or tetragonal lattice. Three of the five markers were "heavy" atoms covalently linked to the structure; namely the iodine and sulfur of the pipsyl group esterified to serine-195 (Sigler *et al.*, 1964, 1966) and the iodine of iodotyrosine-171.

Provided that the appropriate residues are present in both species it is possible that heavy atoms can be used to relate the orientation of highly homologous or mutant molecules occurring in different crystal lattices (Perutz *et al.*, 1964).

Relating the positions of a small number of heavy atoms, as opposed to comparing electron density maps, effects an enormous saving in effort since heavy atoms can be located in projection with little or no phase information, and at a resolution too coarse to recognize most molecular features.

The Use of Iodo-α-chymotrypsin as an Isomorphous Heavy-Atom Derivative. Iodo-α-chymotrypsin was never fully evaluated as an isomorphous derivative of α-chymotrypsin since an excellent map was produced without it (Birktoft *et al.*, 1969). However, an iodinated derivative of γ-chymotrypsin was prepared by Cohen *et al.* (1969), using a method very similar to that introduced by Sigler (1967) and described above. This derivative, which has three sites occupied by 0.86, 0.58, and 0.54 atoms per molecule, has produced useful phases to 2.8-Å resolution (Cohen *et al.*, 1969; D. Davies, personal communication). The value of iodine as a heavy atom in high-resolution phase determination is now well established, particularly where care has been taken to insure a high degree of isomorphism through the use of a "blank ligand." A blank ligand is a moiety which is attached to the parent structure and is similar in every respect to that used to attach the heavy atom, except that the heavy atom is replaced with a light functional group such as methyl group. This approach, introduced in the study of γ-chymotrypsin (Sigler *et al.*, 1964), has been proven successful not only in the X-ray analysis of tosyl-α-chymotrypsin (Matthews *et al.*, 1967; Cohen *et al.*, 1968) but also in ribonuclease (Wykoff *et al.*, 1967) and micrococcal nuclease (Arnone *et al.*, 1969).

General Considerations of Iodinating Crystalline Proteins. From these studies and those on γ-chymotrypsin it can be seen that direct iodination of crystalline proteins is a useful procedure in the crystallographic structure analysis.

It should be noted that when reactions are carried out in the crystal the reaction sites are more likely to be restricted to those which preserve the crystal structure than when carried out in solution. Clearly the crystal structure itself is the best stereochemical deterrent to placing a heavy atom ligand in a position which might disrupt the lattice.

There appears to be no deleterious side reactions or serious disruption of the tertiary structure since, when dissolved and assayed, the iodinated protein retains full enzymatic activity (Table II).

Finally, it should be stressed that when carrying out this reaction in the crystal there is little need to optimize the conditions for iodination of phenols. In particular, high a pH (say greater than 8), which speeds the iodination process but which can also destroy the crystal, is unnecessary since diffusion of I_3^- through the lattice will ultimately be rate limiting in the relative large crystals used for diffraction experiments.

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