ISOLATION AND PARTIAL CHARACTERIZATION OF IODINATED CHYMOTRYPSINOGENS

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Introduction

Chymotrypsinogen contains four tyrosine residues: 94, 146, 171, and 228 (Hartley, 1964; Meloun, Kluh, Kostka, Moravek, Prusik, Vannecek, Keil, and Sorm, 1966). According to Glazer and Sanger (1964), under conditions leading to incorporation of about two gram-atoms of iodine per mole of chymotrypsinogen, only tyrosine 146 is di-iodinated. As Glazer and Sanger pointed out, their analytical procedure was not designed to detect mono-iodotyrosine. That some of the tyrosines in chymotrypsinogen may have been mono-iodinated under their conditions, however, is suggested by studies on iodination of α-chymotrypsin reported by Dube, Roholt, and Pressman (1964, 1966). Even when a total of less than one gram-atom of iodine is bound per mole of α-chymotrypsin, Dube, Roholt, and Pressman observed mono-iodination of tyrosine 146, and to a lesser extent, mono-iodination of tyrosines 94 and 171. In agreement with results for chymotrypsinogen, the tyrosine 146 residue of α-chymotrypsin was di-iodinated when higher (2-3 gram-atoms per mole of protein) levels of iodine were incorporated.

On the basis of these reports, we had hoped by iodination to obtain both a crystallographically identifiable label at tyrosine 146, and one or more heavy-atom derivatives isomorphous with type F crystals of native chymotrypsinogen. Such a label would have greatly facilitated interpretation of already

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existing electron-density maps (Kraut, Sieker, High, and Freer, 1962; Kraut, High and Sieker, 1964).

Unfortunately, we have so far been unable to obtain type F crystals containing iodine, either by direct iodination of pregrown crystals, by crystallization of unfractionated iodinated chymotrypsinogen, or by crystallization of purified fractions. In the meantime, Blow and his colleagues (Matthews, Sigler, Henderson, and Blow, 1967) have determined the three-dimensional structure of α-chymotrypsin at 2 Å, and comparison of this model with our 5 Å chymotrypsinogen map shows that the two molecules are very similar (Kraut, Wright, Kellerman, and Freer, 1967; Wright, Kraut and Wilcox, 1968; Matthews, Cohen, Silverton, Braxton, and Davies, 1968). Consequently, the geometrical location of tyrosine 146 in the zymogen is now clear, and so the iodination project has been discontinued. We are therefore presenting here the results to date of our partial characterization of some of the iodinated chymotrypsinogen fractions isolated in the course of our crystallization studies. The only other such study of isolated fractions of iodinated protein of which we are aware is that of Scheraga and co-workers on iodinated ribonuclease (Woody, Friedman, and Scheraga, 1966; Friedman, Scheraga and Goldberger, 1966).

Methods

Details of all procedures may be found in Weber (1968).

Chymotrypsinogen (Worthington, 5x crystallized) was iodinated in solution by the method of Glazer and Sanger (1964), using ICl and I^{125}-labeled ICl at pH 8.2. In some experiments, however, higher ratios of ICl to protein were employed (up to 6:1) in the hope of forcing iodination of one or both of the histidines.

Analytical polyacrylamide-gel electrophoresis experiments were performed at pH 8.6 in a continuous Tris-HCl buffer (Davis, 1964; Clarke, 1964) using apparatus of the type described by Choules and Zimm (1965).

Chromatography was carried out on a 2.8 x 40 cm column of Whatman CM-52 resin (microgranular carboxymethylcellulose, 1.0 meq./g) equilibrated at 4°C

with 0.005M Tris-HCl buffer, pH 8.5, containing 0.05M sodium acetate. Fractions were eluted with a linear 0.05-0.15M sodium acetate gradient in the same buffer. A flow rate of 12 ml/hr was maintained with a peristaltic pump, and fractions were collected every 30 minutes. Protein peak positions were determined by measurement of absorbance at 282 mμ (A_{282}). The iodine content of each fraction was determined, after gel filtration, by liquid scintillation counting of I^{125}; protein content was determined from both A_{282} and dry weight.

Ratios of di-iodotyrosine to mono-iodotyrosine in all the major iodine-containing chromatographic peaks were also determined. Selected fractions were first subjected to digestion by pronase followed by leucine aminopeptidase, or pepsin followed by chymotrypsin followed by carboxypeptidase A (all enzymes purchased from Worthington). The digests were then analyzed by descending paper chromatography in butanol-acetic acid-water (Katz, Dreyer, and Anfinsen, 1959), and the chromatograms sectioned and counted for I^{125}. Authentic samples of mono-iodotyrosine, di-iodotyrosine, and mono-iodohistidine were also chromatographed in parallel, and these reference chromatograms were used to identify the I^{125}-containing spots.

In separate experiments, the number of di-iodotyrosine residues per mole of protein was also determined for each major iodine-containing chromatographic peak. Di-iodotyrosine (but not mono-iodotyrosine) was first destroyed by performic-acid oxidation, following the method of Hirs (1956). The number of tyrosines destroyed was then estimated by amino-acid analysis of HCl hydrolysates.

The possibility that tryptophan was oxidized during iodination was checked by examining difference spectra, at 250 mμ and 280 mμ, between peptic digests of the iodinated chymotrypsinogen fractions and peptic digests of unmodified chymotrypsinogen (Patchornik, Lawson, Gross, and Witkop, 1960). In another series of experiments, the number of unmodified tryptophans in each fraction was determined (in 8 M urea) by the N-bromosuccinimide titration method of Patchornik, Lawson, Gross and Witkop (1960), as modified by Dus (1967).

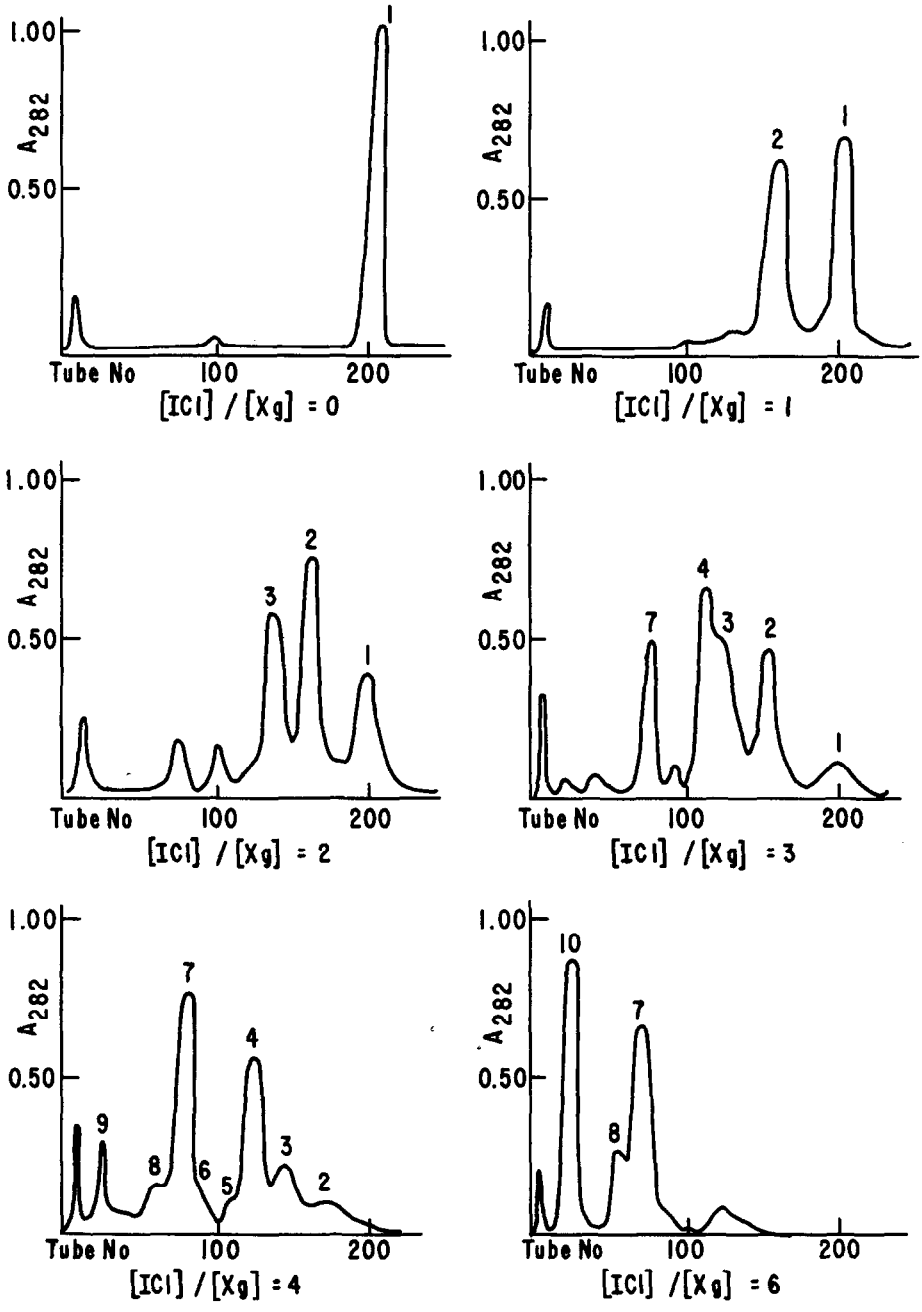
Iodochymotrypsinogen fractions were activated by the method of Jacobsen (1947), and the activity of the resulting iodo-δ-chymotrypsins was determined by the method of Schwert and Takenaka (1955).

Attempted crystallization of iodochymotrypsinogen fractions as type F crystals followed the procedure of Kraut, Sieker, High and Freer (1962). Efforts were also made to iodinated type F crystals of native chymotrypsinogen directly, by adding small aliquots of 0.36M I_3 until the crystals began to crack.

Results and Discussion

As will be seen in Fig. 1, chromatography of ICl-chymotrypsinogen reaction mixtures on microgranular carboxymethylcellulose at pH 8.5 is capable of producing elution patterns containing many well resolved peaks. All except fraction 1 (unmodified chymotrypsinogen) contain iodine. Further, gel electrophoretic patterns of the reaction mixtures are indistinguishable from their chromatographic patterns, and upon subsequent electrophoresis, each individual chromatographic peak migrates as the corresponding electrophoretic band.

Fractions 2,3,4,7,9 and 10 were partially characterized; fractions 5, 6, and 8 could not be obtained in sufficient yields. Results are summarized in Table 1. Fraction 1 is unmodified chymotrypsinogen; its identity was confirmed by running a control chromatogram of the unreacted protein. Fraction 2 appears to contain just one mono-iodotyrosine per molecule. However, our data do not allow us to distinguish whether a single tyrosine is being selectively mono-iodinated, or whether this fraction contains a mixture of mono-iodinated species. The results for fraction 3 are somewhat ambiguous, but suggest that this fraction is a mixture of species, one containing a single di-iodotyrosine, and the other containing two mono-iodotyrosines. Fraction 4 contains one mono-iodotyrosine and one di-iodotyrosine per molecule. Fraction 7 is again apparently a mixture, composed of two components in a three-to-one ratio, the major component containing two di-iodotyrosines per molecule, and the minor component containing one di-iodotyrosine and two mono-iodotyrosines



CMC Chromatograms of Reaction Mixtures for Various Ratios of ICl to Chymotrypsinogen (Xg)

FIGURE 1.

TABLE 1

Fraction	g.atoms I/mole Xg *	moles tyrI/mole Xg †	moles $tyrI_2$/mole Xg †	No. tyr missing ‡
1	0.0 ± 0.1	-	-	0.00 ± 0.05
2	1.1 ± 0.1	0.9 ± 0.1	0.05 ± 0.05	0.00 ± 0.05
3	1.9 ± 0.1	0.7 ± 0.3	0.65 ± 0.15	1.2 ± 0.2
4	3.1 ± 0.1	0.95 ± 0.15	1.05 ± 0.05	1.0 ± 0.1
7	4.0 ± 0.1	0.35 ± 0.15	1.8 ± 0.1	1.6 ± 0.1
9	5.2 ± 0.2	0.9 ± 0.1	2.05 ± 0.1	-
10	5.8 ± 0.1	0.1 ± 0.1	2.95 ± 0.1	2.65 ± 0.15

* Mean of 3-7 independent determinations; uncertainties are mean deviations.

† Mean of duplicate or triplicate determinations for each of the two enzymatic hydrolysis methods; uncertainties are mean deviations.

‡ From amino-acid analyses on performic-acid oxidized fractions. The number of missing tyrosines is equivalent to the number of di-iodotyrosines in the fraction. Uncertainties are mean deviations over 2-4 independent determinations.

per molecule. Fraction 9 contains two di-iodotyrosines and one mono-iodotyrosine per molecule. Because fraction 9 was difficult to isolate in sufficient yield, no performic-acid oxidation experiment was done on it. Fraction 10 contains three di-iodotyrosines per molecule.

A tentative conclusion regarding our separation procedure is that rates of migration are primarily a function of the total number of protein-bound iodines per molecule, rather than of the detailed iodine distribution.

No iodohistidine or oxidized tryptophan was found in any of the fractions, even in the one containing six gram-atoms of iodine per mole of chymotrypsinogen.

Each fraction listed in Table 1 could be activated to δ-chymotrypsin, and the resulting enzyme, in each case, had at least full activity toward N-acetyl-L-tyrosine ethyl ester (ATEE).

All attempts to obtain type F crystals from the various iodinated chymotrypsinogen fractions failed. It is unlikely that the fractionation and concentration procedures were solely responsible, since native chymotrypsinogen, subjected to these procedures, formed type F crystals readily. Further, when type F crystals did grow from an unfractionated reaction mixture, containing about equal amounts of unreacted chymotrypsinogen and mono-iodinated chymotrypsinogen, the crystals proved to consist entirely of unreacted chymotrypsinogen. The mono-iodinated protein was found in the accompanying amorphous precipitate. Finally, when treated with I_3^-, type F crystals of native chymotrypsinogen cracked and became useless after about 5% of the protein was iodinated.

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