IDENTIFICATION OF THE MOST RAPIDLY IODINATING TYROSINE IN SUBTILISIN BPN'

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Introduction

Subtilisin BPN' is an extracellular protease produced by the soil organism Bacillus amyloliquefaciens. It consists of a single 275 residue polypeptide chain. A closely similar protease, subtilisin Carlsberg, is made by the related organism B. subtilis, hence the name. In fact, it was formerly believed that B. amyloliquefaciens was a strain of B. subtilis (Welker and Campbell, 1967). The enzymic properties of the subtilisins are very similar to those of chymotrypsin. The active site of both classes of enzyme contains a reactive serine residue, and the best low molecular-weight substrates and inhibitors for chymotrypsin are also good substrates and inhibitors for the subtilisins. The amino-acid sequences of the subtilisins, however, are totally unrelated to the chymotrypsin sequence.

A crystallographic study of subtilisin BPN' is presently being pursued in this laboratory. For the reasons cited in the preceding communication on chymotrypsinogen, we felt it would also be extremely useful to prepare a selectively iodinated isomorphous derivative of subtilisin BPN'. However, one might have expected considerable difficulty in obtaining such a derivative since subtilisin BPN' contains ten tyrosines (Markland and Smith, 1967), as compared to only four in chymotrypsinogen. Furthermore, unlike chymotrypsinogen, no studies had been previously reported on the relative reactivities

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of the tyrosines in subtilisin, on which to base an expectation that selective iodination would occur. Nevertheless, we have been able, without much difficulty, to obtain crystals of mono-iodinated subtilisin BPN', isomorphous with crystals of the native enzyme, to determine the geometrical location of the bound iodine atom by difference-Fourier methods, and to identify the site of iodination as tyrosine 104 by chemical methods.

Methods

Subtilisin BPN' (Teikoku Chemical Industries) was iodinated by reacting an aqueous solution, containing 60 mg of protein per ml, with varying ratios of ICl or I^{125}-labeled ICl. The pH was adjusted to 9.4 with ammonium hydroxide and maintained with a pH-stat by addition of 0.1 M NaOH. The temperature was 25°C, and time of reaction was 20 minutes. ICl reagent was prepared according to the procedures of Glazer and Sanger (1964), and an equal volume, containing the desired quantity of ICl, was added to the protein solution in small increments throughout the reaction period. The overall amount of iodine bound per molecule of protein was determined, after gel filtration, by liquid scintillation counting of I^{125} and spectrophotometric determination of protein concentration. Under these reaction conditions, about 25% of the total added iodine is actually incorporated into protein.

Analytical polyacrylamide-gel electrophoresis was performed as described in the previous communication, except that a pH 7.8 barbital-NaOH buffer was used. In addition to treating gels with the standard amido-black stain for protein, a second set of parallel gels were also stained for esterase activity toward α-naphthyl acetate (Simms, 1965), and a third parallel set were sliced into 1 mm disks for I^{125} counting.

Activity of subtilisin BPN' and of the iodinated reaction mixtures toward N-acetyl-L-tyrosine ethyl ester (ATEE) hydrolysis was determined by the method of Schwert and Takenaka (1955).

Crystals of iodinated subtilisin BPN' were grown from 30% saturated ammonium sulfate solution following the procedures used by Wright, Alden

and Kraut (to be published) for crystallization of the native enzyme.

A single crystal of iodinated subtilisin BPN' was chosen for 5 Å intensity data collection on a Hilger-Watts automatic diffractometer. Unit-cell parameters were determined by a least-squares fit of observed 2θ values; data collection procedures were the same as those used by Wright, Alden and Kraut. A difference-Fourier map between the iodinated and native subtilisin BPN' was computed using centroid phases for the native enzyme obtained by Wright, Alden and Kraut from multiple isomorphous replacement.

Chemical identification of the labeled tyrosine was carried out by applying the enzymic digestion and chromatographic procedures of Smith and co-workers (Markland, Kreil, Ribadeau-Dumas and Smith, 1966; Markland, Ribadeau-Dumas and Smith, 1967; Kasper and Smith, 1966) to I^{125}-labeled subtilisin BPN'. Since the yield of crystalline mono-iodinated protein was small, and since the reaction mixture from which crystals were grown, in any case, contained only about 5-10% of other iodinated components, it was decided to examine the unfractionated reaction mixture. Duplicate enzymatic digests were subjected to chromatography on a 0.9 x 150 cm Dowex AG-50 column using the gradient-elution procedures of Smith's group. The chromatograms obtained were comparable to those reported by Smith and co-workers. Fractions were assayed for I^{125} by liquid scintillation counting.

Results and Discussion

The most important result of this study, for our x-ray crystallographic investigation, was that mono-iodosubtilisin could be crystallized isomorphously with the native enzyme, directly from an unfractionated reaction mixture. This may be correlated with the observations that only one major iodinated species is produced, and that its enzymic activity appears to be unmodified.

Although subtilisin BPN' contains ten tyrosine residues, the electrophoretic pattern of the unfractionated iodination reaction product is less complex than that of chymotrypsinogen containing only four tyrosines (Weber and Kraut, 1968; Weber, 1968). In addition to a band of unreacted enzyme,

there is always only one major band containing iodine, even when up to four gram-atoms of iodine per mole of enzyme are incorporated. There are also several minor bands containing much higher ratios of iodine to protein than the major band. However, these represent altogether about 5-10% of the total protein when one gram-atom of iodine is incorporated, and even for a reaction mixture in which four gram-atoms of iodine have been incorporated per mole of protein, they represent only 25-35%. These results are in contrast to the dramatic changes observed in the electrophoretic patterns of iodinated chymotrypsinogen reaction mixtures as the overall amount of iodine incorporation was increased.

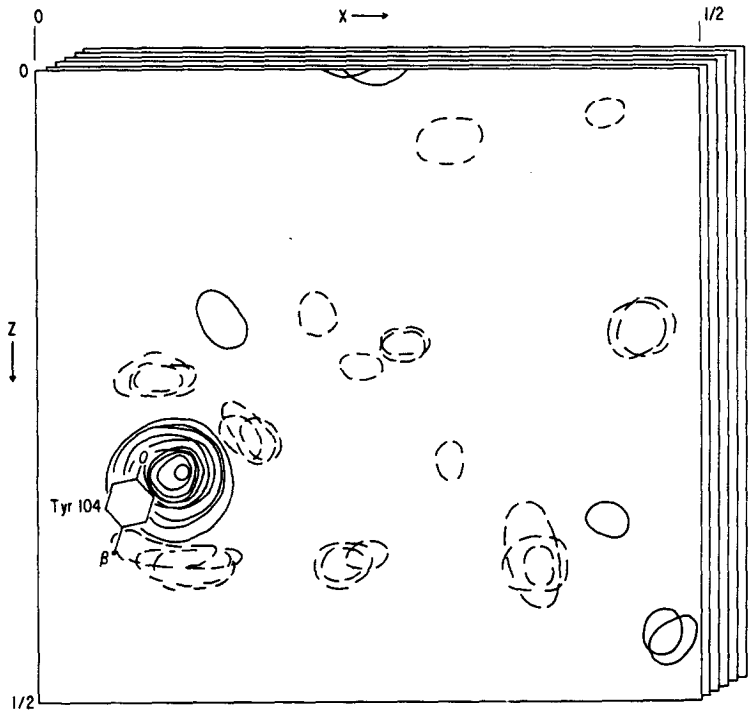
The activity of subtilisin BPN' toward ATEE is unaffected by iodination at the incorporation levels we have examined (up to four gram-atoms per mole). Thus, the enzyme activity of the unfractionated reaction mixtures was in all cases indistinguishable from that of unmodified subtilisin BPN'. Further, each of the product bands in the electrophoretic patterns showed qualitatively normal esterase activity toward the α-naphthyl acetate stain. These results are in agreement with the findings of Johansen, Ottesen and Svendsen (1967) for iodination of subtilisin Novo, which is believed to be identical to subtilisin BPN' (Hunt and Ottesen, 1961; Olaitan, DeLange, and Smith as quoted in Smith, DeLange, Evans, Landon and Markland, 1968).

Crystals were grown from an unfractionated reaction mixture, initially containing 4 moles of ICl per mole of native enzyme, in which 1 gram-atom of iodine per mole of protein had been incorporated overall. The major iodinated electrophoretic component comprised about half of the total protein, the remainder being unmodified protein plus about 5-10% minor iodine-containing components. When these crystals were dissolved and subjected to electrophoretic analysis, a single band was seen in the same position as the major iodinated component of the reaction mixture, and no other bands were observed. The crystals proved to be isomorphous with crystals of native subtilisin BPN' (space group C_2), with $a = 66.8$ Å, $b = 54.4$ Å, $c = 62.8$ Å, $\beta = 92.0^\circ$. These

unit-cell parameters are within 0.2% of those for the native crystal.

A three-dimensional difference-Fourier map, at 5 Å resolution, between iodosubtilisin BPN', crystallized as described above, and native subtilisin BPN' showed a single prominent peak, spherical in shape, and with a height about five times that of the next highest peak, and about ten times the general background level. Its coordinates are x = 0.11, y = 0.77, z = 0.32. Fig. 1 shows a composite of sections y = 37/52 through y = 42/52 of the difference-Fourier map.

The unique site of iodination observed in the difference-Fourier map was identified as tyrosine 104 by partial enzymic digestion of the unfractionated reaction mixture and chromatography of the digest, as described under Methods.



Difference-Fourier map of mono-iodosubtilisin minus native subtilisin at 5 Å resolution; sections y = 37/52 to y = 42/52; electron density in arbitrary units. Contours are drawn at ±75 and at intervals of 150 above 75. The position of tyrosine 104 from the 2.5 Å model is shown in relation to the iodine peak.

FIGURE 1

Chromatograms of the chymotryptic digest showed two I^{125} peaks, the first about twice as high as the second. The first I^{125} peak is at the position of a peptide that Markland, Kreil, Ribadeau-Dumas and Smith (1966) have shown to contain tyrosine 104, but no other tyrosines. The second I^{125} peak does not correspond to any peptide found by Markland et al. (1966), and it would be reasonable to suppose it represents some residual peptide in which cleavage by chymotrypsin has failed to occur at residue 104 because it is now monoiodotyrosine instead of tyrosine. Examination of the peptic and tryptic digests confirmed that tyrosine 104 is the site of iodination. Chromatograms of the peptic digest showed a major I^{125} peak at the position of a peptide containing both tyrosine 104 and tyrosine 91 (Markland, Ribadeau-Dumas and Smith, 1967). For the tryptic digest, on the other hand, no significant I^{125} peaks were seen. Kasper and Smith (1966) have shown that seven of the ten tyrosine residues, including tyrosine 91, occur in the tryptic peptides, but that tyrosines 104, 167, and 171 do not. Accordingly, tyrosine 104 must be the one that is iodinated.

Independent identification of tyrosine 104 as the selectively iodinated residue has recently been provided by the structure determination of subtilisin BPN' at 2.5 Å resolution (Wright, Alden and Kraut, to be published). The coordinates of the iodine peak in Fig. 1 are now seen to lie about 1.4 Å from the presently assumed position of an ϵ-carbon atom of tyrosine 104, well within acceptable limits, at the present stage of the structure analysis, for a covalently bonded iodine atom.

Acknowledgments

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