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# Iodination of a Tyrosyl Residue in Staphylococcal $\alpha$-Toxin†

Paul Cassidy and Sidney Harshman*

ABSTRACT: Iodination of staphylococcal $\alpha$-toxin by the lactoperoxidase method resulted in the maximal incorporation of about 2.5 atoms of iodine per molecule of $\alpha$-toxin. The iodination primarily involved a single tyrosine residue as shown by analysis of both cyanogen bromide and tryptic peptides. Iodination at a level of 1.2 iodine atoms per $\alpha$-toxin molecule led to a dramatic decrease in the hemolytic and lethal activities, although no decrease in the binding of iodinated toxin to rabbit erythrocytes was observed (Cassidy and Harshman (1976), *Biochemistry,* the following paper in this issue). Monoiodinated $\alpha$-toxin was found to have 15% of the specific hemolytic activity of native $\alpha$-toxin. Incubation of rabbit erythrocytes with iodinated $\alpha$-toxin led to a significant protection from the hemolytic activity of native $\alpha$-toxin added later. The results show that modification of a single unique tyrosyl residue in $\alpha$-toxin permits the resolution of $\alpha$-toxin's biological activities from its cell binding activity.

Staphylococcal $\alpha$-toxin, a protein produced in large amounts in the growth medium of *Staphylococcus aureus,* is a hemolytic toxin which is lethal for most mammalian species (Arbuthnott, 1970). Chemical modification techniques which have been used so successfully in the study of structure-function relationships in enzymes (Glazer, 1970) and neurotoxins (Tu, 1973) have not until now been applied to staphylococcal $\alpha$-toxin. In fact, the only reported chemical modification of $\alpha$-toxin involved "toxoiding" or total reaction of the protein with formaldehyde (Bernheimer et al., 1968).

Iodination by the lactoperoxidase method (Morrison et al., 1971) is a protein modification reaction which can be carried out under mild conditions and is a reaction of high specificity. Lactoperoxidase-catalyzed iodination of protein substrates can result in mono or diiodo derivatives of tyrosine and histidine,

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tyrosine being the preferred substrate (Morrison and Bayse, 1973). Iodination can be performed at low concentrations of the oxidant, hydrogen peroxide ($10^{-4}$ M).

Early in the course of this study it was discovered that α-toxin, quantitatively labeled with iodide, lost hemolytic activity. The possibility that the modification of a single amino acid residue in α-toxin could dramatically affect toxic activity was explored.

## Materials and Methods

Lactoperoxidase, pronase CB, and α-chymotrypsin were obtained from Calbiochem. Hydrogen peroxide was an 8.8 M solution in $H_2O$ from Baker Chemical Co. Cyanogen bromide was from Eastman Chemical Co. $Na^{125}I$ was from New England Nuclear. Potassium iodide was from Fisher Scientific Co. Maleic anhydride was from Mallinckrodt Chemical Co. Tos-Phe$CH_2Cl$[1] trypsin was from Worthington Biochemical Corp. The iodide specific electrode was No. F1031 from Radiometer, A.S.

*Iodination of α-Toxin.* Two types of iodinated α-toxin were used in the work described here. α-Toxin trace-labeled to high specific radioactivity (1.5–3.0 μCi/μg) with carrier-free $^{125}I^-$ was used primarily for cell binding studies. In experiments which evaluated the chemical and biological properties of iodinated α-toxin, the toxin was quantitatively iodinated at low specific radioactivity (less than 0.0001 μCi/μg) with a solution of potassium iodide containing $^{125}I^-$.

Iodination of α-toxin was performed either manually (Marchalonis, 1969) or by the potentiometric method (Morrison and Bayse, 1970) using an iodide specific electrode. Manually, 2 mg of α-toxin/ml ($7.2 \times 10^{-5}$ M) in 0.05 M potassium phosphate buffer, pH 7.4, at 25 °C was exposed to 20 μg of lactoperoxidase/ml, varying concentrations of $K^{127}I$ containing $^{125}I^-$ and $H_2O_2$ which was added in small aliquots at 5-min intervals in order to ensure that its concentration in the reaction mixture never exceeded $10^{-4}$ M. Increasing the concentration of $H_2O_2$ above $10^{-4}$ M inhibits lactoperoxidase activity (Morrison and Bayse, 1970).

When the potentiometric method was used, 2–4 mg of α-toxin in 2 ml of 0.05 M potassium phosphate buffer, pH 7.4, was stirred continuously in contact with an iodide specific electrode which was connected to a recording automatic titrator. The titrator was previously calibrated with KI solutions in phosphate buffer and set to maintain an iodide concentration of $2 \times 10^{-6}$ M. The titrator syringe was filled with KI solution (containing $^{125}I$) at a concentration of $1 \times 10^{-3}$ M. Lactoperoxidase was added to a concentration of 10 μg/ml and the iodination reaction was started by manual addition of 1-μl aliquots of $8.8 \times 10^{-3}$ M $H_2O_2$. As iodide was consumed in the reaction, $K^{125}I$ solution was automatically added to the reaction mixture. Scale readings on the automatic titrator were used to calculate the extent of α-toxin iodination. These calculations were confirmed by measuring iodide incorporation into 5% w/v trichloroacetic acid precipitable protein, based on the specific radioactivity of the KI solution.

*Preparation of Monoiodo-α-toxin.* Fifty milligrams of α-toxin was iodinated by the manual method to an average of 0.9 atom of iodide incorporated per molecule of α-toxin. After dialysis for 12 h vs. 4 l. of 0.05 M potassium phosphate buffer, pH 7.4, the iodinated form of the toxin was separated from any contaminating native form by preparative electrophoresis.

Preparative electrophoresis was performed using a Buchler Polyprep apparatus (Buchler Instruments), by the method of Davis (Davis, 1964) as modified by Six and Harshman (1973a). Electrophoresis was performed at pH 8.9 using a 4-cm resolving gel of 7.5% polyacrylamide, 0.8% cross-linked. Fractions of 2.0 ml were collected and aliquots were counted in a well-type γ detector for $^{125}I$. The radioactive protein fractions were pooled, concentrated by pervaporation, and precipitated with 90% saturated ammonium sulfate. The iodide incorporation, based on the specific activity of the $K^{125}I$ solution, was calculated to be 1.1 atoms of iodine per molecule of α-toxin.

*Cyanogen Bromide Cleavage of $^{125}I$-Labeled α-Toxin and Peptide Maleylation.* Eight milligrams of $^{125}I$-labeled α-toxin (0.9 atom of I/α-toxin molecule) in 0.05 M potassium phosphate buffer, pH 7.4, was denatured by heating at 100 °C for 2 min. The precipitate was collected by centrifugation and was dissolved in 3 ml of 70% formic acid. Approximately 10 mg of CNBr was added and the reaction mixture was incubated for 12 h at 25 °C. The reaction was terminated by the addition of 20 ml of distilled $H_2O$ followed by lyophilization. The resulting peptides were maleylated by reaction with maleic anhydride in 0.1 M borate buffer, pH 8.3 (Butler et al., 1969).

*Peptide Mapping.* $^{125}I$-Labeled α-toxin (2 mg) was heat denatured as above and the precipitate collected by centrifugation. The precipitate was suspended in 0.05 M ammonium bicarbonate, pH 8.0, and Tos-Phe$CH_2Cl$ trypsin was added. One percent w/w trypsin was added at 0, 3, and 6 h incubation. After 18 h, the peptide solution was lyophilized. Peptide maps were performed on Whatman 3MM chromatography paper (46 × 57 cm) according to the method described by Bennett (1967). Descending chromatography was performed first for 20 h in the solvent 1-butanol–acetic acid–pyridine–$H_2O$ (90:18:72:60) followed by electrophoresis at 40 V/cm for 3 h at pH 3.5 in pyridine–acetic acid buffer. The papers were dried and peptides detected by ninhydrin spray. $^{125}I$-Labeled peptides were detected by autoradiography using Kodak RP/RZ x-ray film.

*Activity Assays of α-Toxin.* Hemolytic activity was determined as described by Bernheimer (1963). Lethal activity was determined for native α-toxin and $^{125}I$-labeled α-toxin (1.24 atoms of I/α-toxin molecule) by intraperitoneal injection of varying doses of the toxin into mice, 6 mice per dose. $LD_{50}$ values were determined after 24 h. The observed $LD_{50}$ for native α-toxin of 1 μg per mouse is in agreement with published values (Arbuthnott, 1970).

*Other Methods.* Analytical polyacrylamide disc gel electrophoresis was performed according to the method described above for preparative electrophoresis using 5 × 90 mm gels. Protein was determined by the fluorescamine reaction (Udenfriend et al., 1972) using bovine serum albumin as a standard. α-Toxin was prepared as described previously (Six and Harshman, 1973a).

## Results

*Iodination of α-Toxin.* When staphylococcal α-toxin is incubated with a threefold molar excess of potassium iodide in the presence of lactoperoxidase and hydrogen peroxide, the protein is iodinated, resulting routinely in an 80–90% drop in its hemolytic activity (Table I). Incubation of the protein with KI or $H_2O_2$ in the presence of lactoperoxidase does not result in inactivation, indicating that the inactivation with all reagents present is specifically caused by enzyme-catalyzed iodination. Under these reaction conditions, an average of 1.6 atoms of iodide was incorporated per molecule of α-toxin. If the iodination reaction is followed kinetically by the use of the iodide

[1] Abbreviations used: CNBr, cyanogen bromide; Tos-Phe$CH_2Cl$, L-1-tosylamido-2-phenylethyl chloromethyl ketone;

TABLE I: Iodination of α-Toxin.

| Reaction Mixture[a] | | Hemolytic Units/mg |
|---|---|---|
| α-Toxin | (35 nmol) | 23 800 |
| α-Toxin<br>Lactoperoxidase<br>$H_2O_2$ | (35 nmol)<br>(20 μg)<br>(100 nmol) | 23 300 |
| α-Toxin<br>Lactoperoxidase<br>KI | (35 nmol)<br>(20 μg)<br>(100 nmol) | 24 000 |
| α-Toxin<br>Lactoperoxidase<br>KI<br>$H_2O_2$ | (35 nmol)[b]<br>(20 μg)<br>(100 nmol)<br>(100 nmol) | 1 400 |

[a] Conditions of the reaction were: 0.05 M potassium phosphate buffer, pH 7.4, 1 ml, 25 °C, 30 min. [b] Iodide incorporation was 1.6 atoms of I/α-toxin molecule.

specific electrode, about 1 atom of iodide reacts per molecule of α-toxin rapidly. If the reaction is allowed to continue, an additional 1.5 atoms of iodide reacts but at a much slower rate. After a total of 2.5 atoms of iodide is incorporated per molecule of α-toxin, the reaction essentially ceases.

*Polyacrylamide Gel Analysis of Iodinated α-Toxin.* Analysis of α-toxin preparations iodinated to varying degrees shows that the monoiodinated form of α-toxin can be resolved from the native form (Figure 1). Moreover, the amount of the faster-moving, monoiodinated form is directly proportional to the amount of iodide incorporated. The preparation of α-toxin that had incorporated 1.24 atoms of I per α-toxin molecule showed a faint band that migrated farther than the major monoiodinated band and presumably is the diiodo form of α-toxin. These results indicate that the incorporated iodide is uniformly distributed through the population of α-toxin molecules and is not accumulating in a subpopulation of denatured molecules.

*Effect of Iodination on Biological Activity.* To evaluate the effect of iodination on the biological properties of α-toxin, the hemolytic, lethal, and the rabbit-erythrocyte-binding activities were compared. The activities were measured after the incorporation of 0.002, 0.34, 0.71, and 1.24 atoms of iodide per molecule of α-toxin. As the amount of iodide increased, both the hemolytic and lethal activities fell in parallel such that, at an average incorporation of 1.24 atoms of iodide per molecule of α-toxin, only 10% of the native activity remained. In contrast to these findings, the capacity of the iodinated α-toxin to bind to rabbit erythrocytes remained indistinguishable from that of native toxin.

*Hemolytic Activity of Monoiodinated α-Toxin.* Since monoiodinated α-toxin retained a small amount of hemolytic activity, we next determined if this activity could be assigned to small amounts of residual unmodified α-toxin. To this end we subjected a 50-mg preparation of iodinated α-toxin (0.9 atom of I/α-toxin molecule) to preparative gel electrophoresis (see Materials and Methods). The isolated monoiodo form of α-toxin contained 1.1 atoms of I/α-toxin molecule confirming that resolution of the native and monoiodo forms of α-toxin had been achieved. When the isolated monoiodo α-toxin was compared with native toxin, it still showed 15% of the specific activity of native toxin, indicating that monoiodinated α-toxin has intrinsic residual hemolytic activity.

To confirm that the monoiodinated α-toxin was free of any

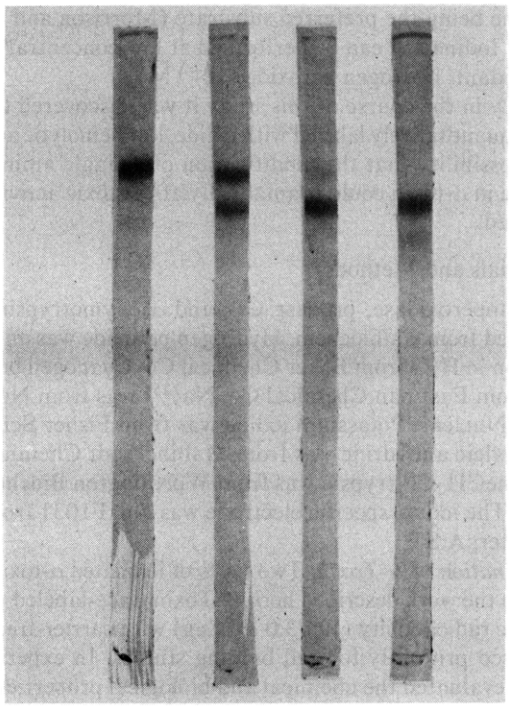

FIGURE 1: Polyacrylamide gel electrophoresis at pH 8.9 of iodinated α-toxin at different levels of iodide incorporation. Twenty micrograms of total protein was applied to each gel. The levels of iodide incorporation (I atoms per α-toxin molecule) were, from left to right: 0.002, 0.34, 0.71, and 1.24.

contaminating native toxin, samples of native α-toxin were electrophoresed in the pH 8.9 analytical system. After 3 h of electrophoresis, the polyacrylamide gels were removed and sliced into 1-mm slices, and the slices were eluted overnight at 4 °C in 0.1 ml of phosphate-buffered saline containing 1 mg/ml bovine serum albumin. Small aliquots of eluate were then used to determine both the presence of the radioactive protein and hemolytic activity.

The purified monoiodinated α-toxin preparation (Figure 2, bottom) contained hemolytic activity which coelectrophoresed with the radioactive protein peak. No hemolytic activity peak was detected in the region of the gel corresponding to native α-toxin (Figure 2, top). Coelectrophoresis of mixtures of native and monoiodinated α-toxin (data not shown) confirmed that the two species could be resolved by this method. Thus residual hemolytic activity in the monoiodinated α-toxin preparation appears to be a property of the modified toxin itself.

*Identification of Iodinated Amino Acid Residues.* To identify the iodinated amino acid residues in $^{125}$I-labeled α-toxin, a trace-labeled $^{125}$I-labeled α-toxin preparation and an α-toxin preparation which had incorporated 1.6 iodide atoms per α-toxin molecule were digested with a pronase CB–α-chymotrypsin mixture. The resulting digests were lyophilized, redissolved in 50% aqueous pyridine and chromatographed on thin-layer cellulose medium in 1-butanol–acetic acid–water (4:1:2). The system resolves standard monoiodotyrosine, diiodotyrosine, and monoiodohistidine. After development, the thin-layer sheet was dried, and the standard amino acids were detected by ninhydrin spray. The sheet was then cut into 5-mm strips and the strips were counted for $^{125}$I.

Figure 3 shows that only tyrosine was iodinated in either $^{125}$I-labeled α-toxin preparation, with the exception of a trace of monoiodohistidine in the 1.6 atoms of I/α-toxin molecule,

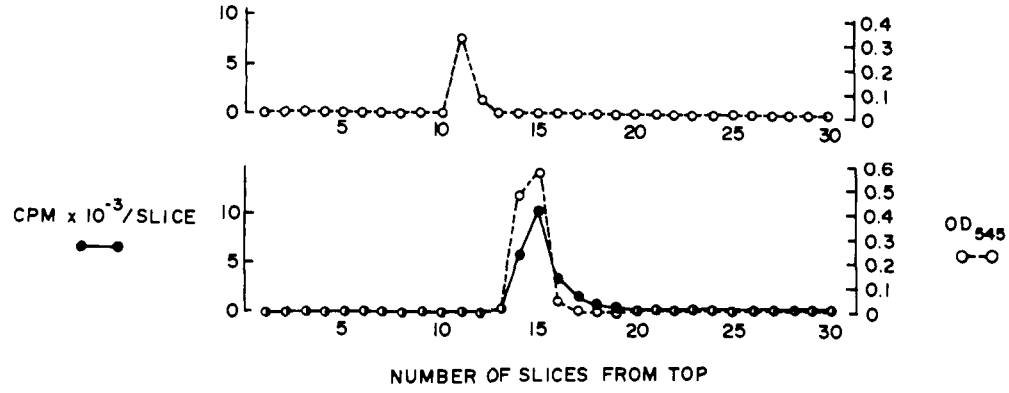


FIGURE 2: Simultaneous detection of $^{125}I$ radioactivity and hemolytic activity on polyacrylamide gel electrophoresis. The gels were run at pH 8.9. Native α-toxin and purified monoiodo-α-toxin (1.1 atoms of I/α-toxin molecule) were used and 1.0-mm slices were assayed (see Materials and Methods). Only the first 30 gel slices are shown. In gel 1, 20 μg of native α-toxin was applied and 2-μl samples of the phosphate-buffered saline eluate were tested for hemolytic activity. In gel 2, 20 μg of monoiodinated α-toxin was applied and 10-μl-samples of the phosphate-buffer saline eluate were tested for hemolytic activity. Left scale represents $^{125}I$ counts per minute × $10^{-3}$ per total gel slice (●—●); right scale represents $OD_{545}$ of hemolysate supernatants per aliquot tested (O - - O).

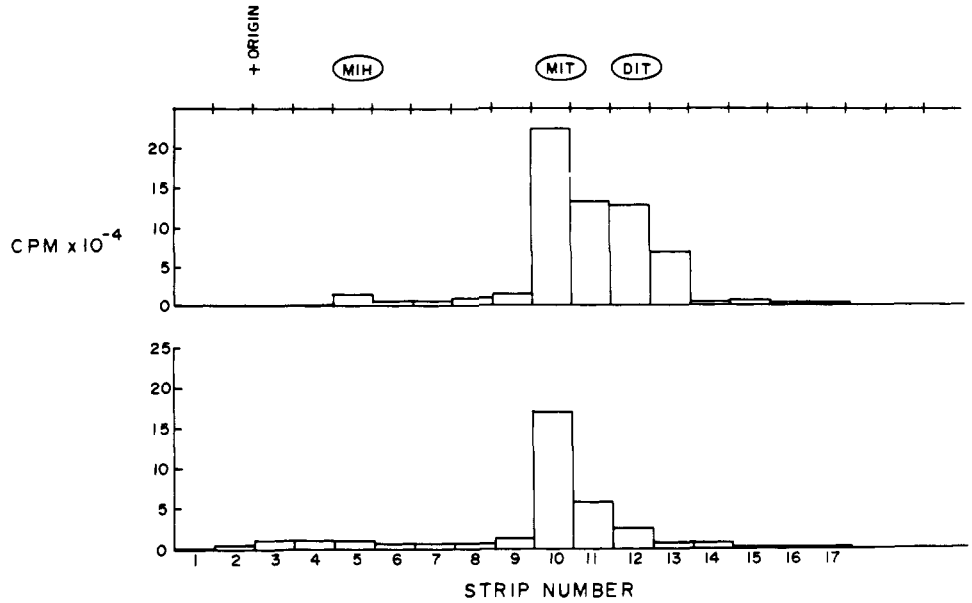


FIGURE 3: Thin-layer chromatography of pronase CB, α-chymotrypsin digests of $^{125}I$-labeled α-toxin. (Top) α-Toxin iodinated to an average of 1.6 I atoms per α-toxin molecule. (Bottom) α-Toxin iodinated to an average of 0.34 I atom per α-toxin molecule. Standard amino acids shown above are monoiodohistidine (MIH), monoiodotyrosine (MIT), and diiodotyrosine (DIT). Pronase CB and α-chymotrypsin were both present as 1% w/w relative to α-toxin protein, and digestions were carried out for 18 h at 25 °C, in 0.05 M potassium phosphate buffer, pH 7.5.

$^{125}I$-labeled α-toxin preparation. Approximately 90% of the radioactive amino acid residues were monoiodotyrosine in the trace-labeled preparations, and 10% could be ascribed to diiodotyrosine (Figure 3, bottom). The 1.6 atoms of I/α-toxin molecule preparation contained approximately 70% monoiodotyrosine and 30% diiodotyrosine (Figure 3, top).

*Cyanogen Bromide Digest of Iodo-α-toxin.* α-Toxin iodinated to 0.9 g-atom of iodide per mol of α-toxin was reacted with cyanogen bromide in 70% formic acid and the resulting peptides were maleylated. The peptides were passed through a Sephadex G-50F column (5 × 83 cm) in 0.1 M ammonium acetate buffer, pH 7.4. Figure 4 represents the elution pattern of the CNBr peptides (detected by absorption at 225 nm) and the $^{125}I$-labeled material. Over 85% of the radioactivity eluted in the position of CNBr peptide IV in the terminology of Six and Harshman (1973b). After digestion with the pronase–chymotrypsin mixture, amino acid analysis showed the presence of monoiodotyrosine, a trace of diiodotyrosine, and an unidentified radioactive product (Figure 5). These results confirm those obtained following digestion of whole iodinated α-toxin (Figure 3, above) and locate the tyrosine residue to a single CNBr peptide.

*Tryptic Peptide Maps of $^{125}I$-Labeled α-Toxin.* Since CNBr digests of iodo-α-toxin revealed that iodide was being incorporated into a specific region in α-toxin, the iodinated protein was digested with Tos-Phe$CH_2Cl$ trypsin and the resulting peptides were mapped by high-voltage electrophoresis and chromatography on paper. Figure 6 represents reconstruction of the peptide maps obtained from native α-toxin (Figure 6, top) and iodinated α-toxin (0.9 atom of I/α-toxin molecule; Figure 6, middle). It can be seen that greater than 90% of the iodine (detected by autoradiography of $^{125}I$ comigrates with a single ninhydrin-positive tryptic peptide. The position of the uniodinated counterpart peptide is indicated by the dotted circle. Figure 6 (bottom) shows that it is possible to lable more than one tryptic peptide if the toxin is first di-

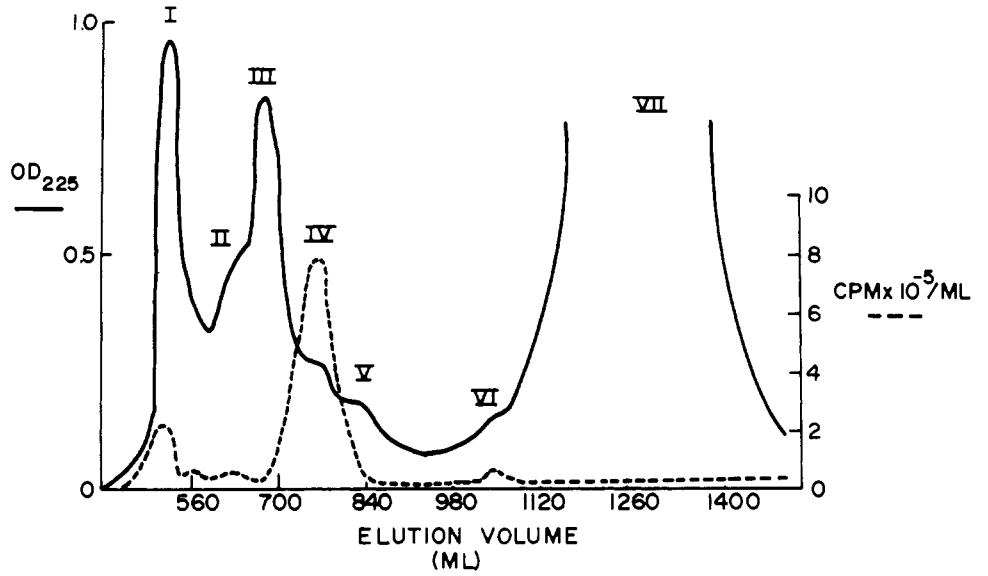


FIGURE 4: Chromatography of CNBr peptides of $^{125}$I-labeled $\alpha$-toxin on Sephadex G-50G. Designation of peptides is from Six and Harshman (1973a,b). For further discussion, see text.

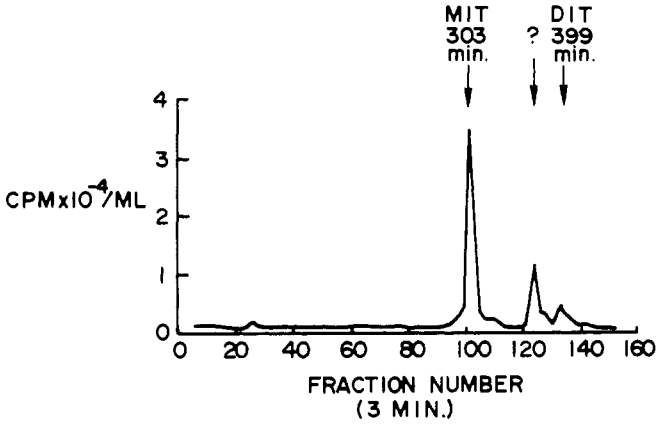


FIGURE 5: Amino acid analysis of a Pronase CB-$\alpha$-chymotrypsin digest of CNBr peptide IV from $^{125}$I-labeled $\alpha$-toxin. Amino acid analysis was performed on a Technicon analyzer using Type C2 resin. Elution of standard amino acids including monoiodotyrosine (MIT) and diiodotyrosine (DIT) was monitored by ninhydrin reaction and simultaneous collection of fractions for detection of $^{125}$I. [$^{14}$C]Valine was used to align the ninhydrin and radioactive elution profiles. Digestion was performed as in Figure 3.

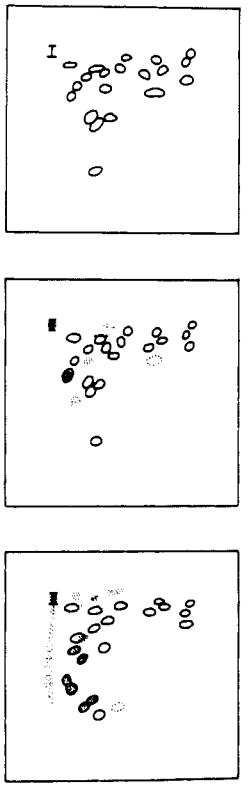


FIGURE 6: Tryptic peptide maps of $\alpha$-toxin and $^{125}$I-labeled $\alpha$-toxin. (Top) Native $\alpha$-toxin. (Middle) $^{125}$I-Labeled $\alpha$-toxin (0.9 I atom per $\alpha$-toxin). (Bottom) Native $\alpha$-toxin after trypsin digestion and subsequent iodination. Electrophoresis is from left to right at pH 3.5. Chromatography is from top to bottom. Radioactivity detected by autoradiography is indicated by stippling. Ninhydrin positive spots are shown by solid circles. The original position of the major iodinated peptide is shown by the dotted circle (Figure 5, middle).

gested with Tos-Phe$CH_2$Cl trypsin and then iodinated. In this case six major ninhydrin-positive tryptic peptides become labeled with $^{125}$I (Figure 6, bottom).

The major $^{125}$I-labeled tryptic peptide (Figure 6, middle) was isolated from two tryptic maps of $^{125}$I-labeled $\alpha$-toxin (4 mg total protein) by cutting the radioactive spot and eluting the peptide with $H_2O$ from the paper. After hydrolysis in 6 N HCl for 20 h, which is known to regenerate tyrosine from iodotyrosine, the amino acid composition was determined (Table II). The $^{125}$I-labeled tryptic peptide was a lysine peptide, which contained one tyrosine. Its content of hydrophobic amino acids was not abnormally high. The composition of the $^{125}$I-labeled tryptic peptide is consistent with the peptide's origin being in an amino acid sequence inside CNBr peptide IV, with the exception of valine which, unaccountably, is higher in the $^{125}$I-labeled tryptic peptide. Studies to sequence $\alpha$-toxin are in progress and should lead to a resolution of this paradox.

*Effect of Preincubation of Rabbit Erythrocytes with Monoiodo-α-toxin on Their Hemolytic Sensitivity to Native α-Toxin.* To test if the monoiodotoxin binds at the same sites on the rabbit erythrocyte as does the native form of $\alpha$-toxin, a direct competition experiment for binding sites was done. Since monoiodotoxin has only 15% of the native $\alpha$-toxin's specific hemolytic activity, it should be possible to demonstrate protection of cells preincubated with monoiodo-$\alpha$-toxin from

TABLE II: Amino Acid Composition of the $^{125}$I-Labeled Tryptic Peptide of $^{125}$I-Labeled α-Toxin.

| | $^{125}$I-Labeled Tryptic Peptide | CNBr Peptide IV[a] |
|---|---|---|
| Aspartic acid | 3.1 | 5.0 |
| Thr | 2.7 | 3.7 |
| Ser | 1.4 | 1.7 |
| Glutamic acid | 0.4 | 1.0 |
| Pro | | 0.5 |
| Gly | 3.3 | 2.7 |
| Ala | 1.1 | 1.1 |
| Val | 3.1 | 1.1 |
| Ile | 1.4 | 1.7 |
| Leu | 1.0 | 1.0 |
| Tyr | 0.6 | 0.6 |
| Phe | | 0.6 |
| Lys | 1.0 | 2.0 |
| His | | |
| Arg | | |
| Met | | |
| Hse | | 1.0 |

[a] CNBr peptide IV composition from Six and Harshman (1973b). The values underlined were assigned as integers.

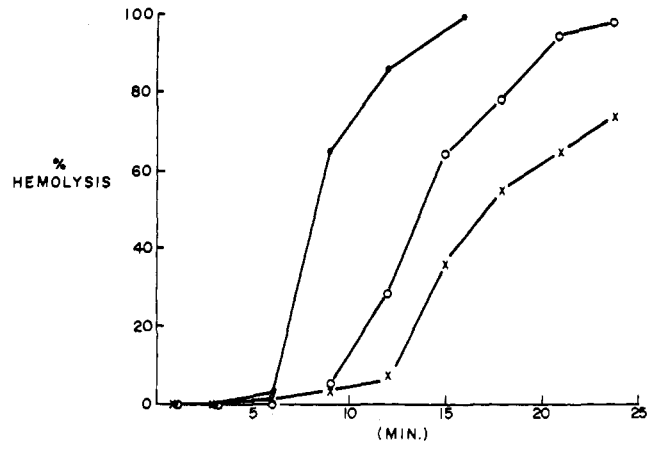


FIGURE 7: Kinetics of α-toxin-induced hemolysis of rabbit erythrocytes in the presence of monoiodo-α-toxin. In the incubations which contained native α-toxin, zero time indicates the time of addition of native α-toxin. In the incubations which contained monoiodo-α-toxin, monoiodo-α-toxin was added 10 min before zero time. (●) Percent hemolysis in the presence of 1 μg/ml α-toxin. (X) Percent hemolysis, 0.7 μg/ml monoiodo-α-toxin preincubated 10 min. (○) Percent hemolysis, 0.7 μg/ml monoiodo-α-toxin preincubated 10 min, 1 μg/ml α-toxin.

the hemolytic effect of native toxin. Figure 7 shows the results of such an experiment. Rabbit erythrocytes exposed to 0.7 μg/ml of the monoiodo-α-toxin for 10 min at 25 °C are lysed with a significantly increased lag time before hemolysis begins relative to cells which were not preincubated with monoiodo-α-toxin. This result suggests that the monoiodinated α-toxin occupies the same sites as does native α-toxin and is thus an appropriate probe for studying α-toxin binding.

## Discussion

Although the mechanism by which α-toxin damages membranes is unknown, it is clear that the chemical modification of a single tyrosyl residue in the toxin can greatly decrease both the hemolytic and lethal activities of the protein. Since the reactive tyrosine is iodinated by lactoperoxidase reaction, in contrast to the other 9-10-tyrosyl residues in native α-toxin, it must be a "surface" tyrosine, available for reaction with the lactoperoxidase marcomolecule. Limited trypsin digestion of undenatured iodinated α-toxin shows that the iodinated peptide is released early in the digestion, again suggesting that it is relatively exposed on the toxin's surface (Cassidy, unpublished observation).

Loss of biological activity of a protein through the modification of a single tyrosyl residue is not common, although a few such reports exist in the literature. Staphylococcal nuclease has been nitrated at tyrosine 85 with a complete loss of catalytic activity toward DNA and RNA (Cuatrecasas et al., 1968). A microbial aminopeptidase and pig-kidney aminopeptidase both contain an especially reactive tyrosine (one residue per subunit; Wachsmuth, 1967a,b). Iodination of the tyrosyl residue results in a complete loss of peptidase activity. Iodination of tyrosine 248 of carboxypeptidase A results in a loss of peptidase activity and an increase in esterase activity of five- to six-fold (Simpson and Vallee, 1966). The more usual finding is that progressive iodination or nitration of tyrosyl residues in a protein leads to a gradual loss of biological activity. Examples of relatively nonspecific loss of activity on tyrosine modification are the iodination of levansucrase (Rapport et al., 1967) whose inactivation is complete after 14 to 15 iodide atoms have been incorporated or the nitration of ovotransferin (Tsao et al., 1974) whose iron binding activity is lost after the modification of 6 to 7 surface tyrosines.

Speculation concerning the role of the reactive tyrosine in α-toxin is difficult since no catalytic activity has been positively related to the toxin's hemolytic or lethal activities, and indeed the toxin activity may not involve the breaking of covalent bonds. The differential effect of iodination on the binding of the toxin to rabbit erythrocyte membranes (Cassidy and Harshman, 1976) and on its hemolytic and lethal activities suggests that binding and biological activity are functionally separate processes. The iodination of model compounds has shown that the phenoxyl p$K$ may be lowered by one or two pH units by the formation of monoiodotyrosine (Edelhoch, 1962; Covelli and Wolff, 1966). The lowering of the phenoxyl p$K$ would reduce capacity for formation of hydrogen bonds with a substrate molecule, reducing catalytic activity. Iodination also introduces into the tyrosyl residue the iodine atom which has an atomic radius of about the same size as the aromatic ring. The presence of such a bulky group might prevent, by steric considerations, the physical interaction of the tyrosine ring with a substrate molecule.

Preliminary experiments (unpublished) indicate that nitration of α-toxin with tetranitromethane (Riordan and Vallee, 1972) can also destroy the hemolytic activity of the toxin. However, significant cross-linking of the toxin occurs in the nitration reaction as has been observed with other proteins (Doyle et al., 1968; Vincent et al., 1970). Thus the interpretations of these complex results are not straightforward and require further study.

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# Studies on the Binding of Staphylococcal $^{125}$I-Labeled $\alpha$-Toxin to Rabbit Erythrocytes†

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ABSTRACT: Staphylococcal $\alpha$-toxin, a hemolytic exotoxin, can be iodinated using the lactoperoxidase method. $^{125}$I-Labeled $\alpha$-toxin binds to rabbit erythrocytes in an apparently irreversible and highly specific manner. The binding of $^{125}$I-labeled $\alpha$-toxin to erythrocytes of rabbit and human reflects the species specificity of native $\alpha$-toxin. Binding of $^{125}$I-labeled $\alpha$-toxin is blocked by the presence of native $\alpha$-toxin, $^{127}$I-labeled $\alpha$-toxin, or anti-$\alpha$-toxin antibody. Simultaneous assays of $^{125}$I-labeled $\alpha$-toxin binding and leakage of intracellular $^{86}Rb^+$ suggest that toxin binding and membrane damage are separate, sequential functions. Both the rate and extent of binding are temperature dependent. Rabbit erythrocytes possess $5 \times 10^3$ binding sites/cell, while human erythrocytes possess no detectable binding sites. Treatment of rabbit erythrocytes with $^{125}$I-labeled $\alpha$-toxin appears to decrease the number of unoccupied binding sites. Chaotropic ions can inhibit $^{125}$I-labeled $\alpha$-toxin binding and cause bound $^{125}$I-labeled $\alpha$-toxin to dissociate from rabbit erythrocyte membranes. Treatment of intact rabbit erythrocytes with pronase reduces both the binding capacity of the cells for $^{125}$I-labeled $\alpha$-toxin, and the cells' sensitivity to hemolysis by native $\alpha$-toxin. It is proposed that the primary binding site for $\alpha$-toxin in biomembranes is a surface membrane protein.

A number of proposals have been made concerning the interaction of staphylococcal $\alpha$-toxin with erythrocyte membranes and the mechanism of toxin-induced hemolysis. It has been proposed that $\alpha$-toxin is a protease having activity for membrane proteins (Wiseman and Caird, 1972), and that the toxin is an esterase having specificity for cholesterol esters (Harvie, 1974). Weissman et al. (1966), Buckelew and Colacicco (1971), and Freer et al. (1973) have proposed that disruption of susceptible biomembranes may be explained solely by a direct interaction with membrane lipid. This proposal has been based on the interaction of $\alpha$-toxin with artificial lipid dispersions (Weissman et al., 1966; Freer et al., 1973) and with lipid monolayers (Buckelew and Colacicco, 1971). That homogeneous $\alpha$-toxin does release internal marker from liposomes has been confirmed recently (Cassidy et al., 1974). Although erythrocytes of different species vary in their sensitivity to the toxin, no such variation in sensitivity was found in liposomes derived from membrane lipid extracted from erythrocytes of different species. Also, the binding of radiolabeled $\alpha$-toxin to erythrocytes has been found to reflect the species specificity of the native toxin (Cassidy and Harshman, 1973).

In a preliminary report (Cassidy and Harshman, 1973) we concluded that the binding of $^{125}$I-labeled $\alpha$-toxin to rabbit erythrocytes was complex in that an apparent decrease in

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