Heterogeneity of Chloramine T- and Lactoperoxidase-Radioiodinated Human Calcitonin

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Radioiodination reportedly damages peptides, but the nature of the damage has not been adequately examined. Utilizing isoelectric focusing, we examined the products of Chloramine T- and lactoperoxidase-directed radioiodinations of human calcitonin. Initially, the reaction products were purified by adsorption onto and elution from microfine silica (QUSO-G32). Radioiodination of the calcitonin by Chloramine T and lactoperoxidase produced a heterogeneous population of $^{125}$I-labeled peptides exhibiting apparent isoelectric points that were more acidic than that of unlabeled synthetic calcitonin. Variation in the products among radioiodinations and the inability of QUSO-G32 to resolve the components of the reaction mixture prompted our examination of alternative purification procedures. Anion-exchange chromatography on QAE-Sephadex effectively separated $^{125}$I diiodotyrosine containing calcitonin from free iodine and $^{125}$Iiodolactoperoxidase. Our data indicate that: (a) radioiodination of human calcitonin by Chloramine T and lactoperoxidase induced alteration in the peptide as evidenced by isoelectric point, (b) specific $^{125}$Iiodopeptides vary in incidence and relative abundance among radioiodinations, (c) identification of the labeled amino acid in $^{125}$Iiodopeptides cannot ensure integrity of the molecule, and (d) isoelectric focusing provides a method of comparing the products of peptide radioiodinations among laboratories.

Additional Keyphrases: isoelectric focusing • nature of radiation damage to peptides

Radioiodination demonstrably decreases the biological activity (1, 2) and alters the electrophoretic mobility (3-5) of hormones. These observations indicate that the basic structure of some of the hormone molecules in the reaction mixture had been dramatically altered and therefore the contents of the reaction would correspond to a heterogeneous population of $^{125}$Iiodopeptides. Use of a heterogeneous labeled peptide preparation could adversely affect the accurate determination of in vivo plasma half-life, metabolic clearance rate, and tissue distribution. In addition, the heterogeneous preparation of $^{125}$Iiodopeptides would create multiple association/dissociation constants (6, 7) in a radioimmunoassay, which may confound data interpretation.

Until now, the products of peptide radioiodinations have not been adequately examined for heterogeneity. In this report, we examined by isoelectric focusing the products of Chloramine T- and lactoperoxidase-directed radioiodination of synthetic human calcitonin. The variability of the $^{125}$Iiodopeptides observed among the radioiodinations prompted the development of procedures that would reproducibly yield high-quality $^{125}$I-labeled human calcitonin.

Materials and Methods

Radioiodinations and Fractionation of $^{125}$Iiodopeptides

The iodination reactions were performed in "V-vials" (Kontes, Vineland, NJ 08360; cat. no. K-749001) behind a 30.7 x 30.7 x 2.56 cm leaded glass shield (Nuclear Pacific, Inc., Seattle, WA 98108) in a charcoal-filtered fume hood. The V-vials were sealed with a silicone rubber septum, and Hamilton (Reno, NV 89510) microsyringes were used for manipulation of reactants. Synthetic human calcitonin was provided by Ciba-Geigy, Ltd., Basel, Switzerland.

Radioiodinations with Chloramine T (Eastman Kodak Co., Rochester, NY 14650) were performed at pH 6.5 in 0.5 mol/L sodium phosphate buffer. The sodium $^{125}$iodide was from Amersham (cat. no. 1MS.300; Arlington Heights, IL 60005). Molar ratios of reactants were 1/1/15.8/1.9 for iodine/calcitonin/Chloramine T/sodium metabisulfite. Calcitonin (1μg) was exposed to Chloramine T for 2 min in a volume of 45 μL. Lactoperoxidase (EC 1.11.1.7, lot no. 200141; Calbiochem, LaJolla, CA, 92112) directed radioiodinations were performed at pH 5.0 in 0.4 mol/L sodium acetate. We added 4 μg of calcitonin, 3 μg of enzyme (3 μg), and 1 mCi of sodium $^{125}$iodide, the final volume being 40 μL. Freshly prepared hydrogen peroxide (10 μL, 6 mg/L; J. T. Baker Chemical Co., Phillipsburg, NJ 08865) was added at 5-min intervals. The reaction was terminated at 10 min by adding sodium azide (200 μL, 25 mmol/L).

The $^{125}$Iiodopeptides were fractionated on QUSO G-32 (Philadelphia Quartz Co., Valley Forge, PA 19482) and QAE-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, NJ 08854). The iodination reaction mixtures were placed in polypropylene tubes containing 3 mg of QUSO suspended in water (8), centrifuged, and the supernates discarded. The $^{125}$Iiodopeptides were eluted from QUSO with a mixture of acetone/acetic acid/water (20/1/79 by vol). QAE-Sephadex A-25 was swollen in 25 mmol/L sodium azide, rinsed with distilled de-ionized water, and added to disposable columns (1 x 5 cm, cat. no. QS-Q; Isolab, Inc., Akron, OH 44321). Two milliliters of the acid–acetone eluent from QUSO was diluted to 15 mL, adjusted to pH 10.5 and applied to the column. The columns were rinsed with water and then eluted with a 20-mL gradient of 0.0-0.1 mol/L tris(hydroxymethyl)aminomethane, pH 10.5. $^{125}$Iiodopeptides were eluted with a 0.0-0.3 mol/L sodium chloride gradient in 0.1 mol/L tris(hydroxymethyl)aminomethane, pH 10.5.

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Identification of [125I]iodopeptides were incubated with 400 μg of pronase (a mixture of enzymes EC 3.4.21.4 and 3.4.24.4) in 0.1 mol/L ammonium bicarbonate, pH 7.9, for 72 h at 37 °C, with constant shaking. The digest was extracted with butanol/hydrochloric acid (0.1 mol/L), 2/0.1 by volume, and the organic phase evaporated under nitrogen. The residue was dissolved in ethanol/hydrochloric acid (0.1 mol/L), 2/1 by volume, and applied to silica gel thin-layer Chromograms (cat. no. 13179, Eastman Kodak Co.). We applied nonradioactive monoiodotyrosine, diiodotyrosine, and diiodohistidine in separate lanes. Chromatograms were developed (9) in benzyl alcohol/acetonitrile ammonium hydroxide (1 mol/L), 1/4/1 by volume, the standards were located by exposure to iodine vapor, and the strip containing the radioactivity was serially sectioned (0.5 cm) for quantification in a Searle Analytical gamma counter (Model 1185; G. O. Searle & Co., Des Plaines, IL 60018).

Isoelectric Focusing

For these experiments we used a 110-mL isoelectric focusing column (Model 8100-1; LKB Instruments, Inc., Rockville, MD 20852) maintained at 5–7 °C.

In most analyses the column was established with the acid electrolyte solution in contact with the lower anode. The lower acid electrolyte solution was prepared by dissolving 15 g of sucrose in 12 mL of water and 4 mL of 1 mol/L phosphoric acid. The linear sucrose gradient was prepared on top of the lower electrolyte solution by appropriately mixing light and dense sucrose solutions. The light sucrose solution was composed of 2.7 g of sucrose, 0.7 mL of pH 3.5–10 Ampholine, and 52 mL of water. The dense sucrose solution was prepared by dissolving 27 g sucrose in a final volume of 52 mL water and adding 2 mL of pH 3.5–10 Ampholine. We added samples to the dense sucrose solution before establishing the linear gradient. The basic electrolyte solution consisted of 2.6 mL of 1 mol/L sodium hydroxide diluted to 10 mL with water. In those columns where the lower electrolyte solution was basic, the polarity was reversed and 15 g sucrose was added to the sodium hydroxide solution. The LKB Model 2103 power supply was set to deliver a constant current of 3 mA and maximum power of 1 W.

At the end of each experiment, generally 66 to 70 h, water was pumped (65 mL/h) onto the upper electrode solution, and 1-mL aliquots were collected from the bottom of the column. Fractions were maintained at 4 °C while the pH profile was determined with a pH meter (Radiometer, Model 26; The London Co., Cleveland, OH 44145).

Radioimmunossay

The radioimmunoassay was established as a nonequilibrium double-antibody system (0.5 mL final volume) in which we used successive incubations for 48 h (sample + rabbit anti-human calcitonin serum), 72 h (radiolabeled calcitonin added), and 24 h (goat anti-rabbit gamma globulin added). The final dilution of the rabbit anti-human calcitonin serum in the assay tube was 1/35 000. The assay buffer contained, per liter, 1 g of bovine serum albumin, 0.05 mol of phosphate, 0.15 mol of sodium chloride, 0.1 g of sodium azide, and 0.1 g of ethylenediaminetetraacetate; its pH was 7.5. The rabbit anti-human calcitonin serum (1000) was drawn from rabbits immunized with unconjugated synthetic human calcitonin (Ciba-Geigy).

The rabbit anti-human calcitonin serum exhibits specificity to the amino-terminal ring structure of calcitonin. Examination of sera from 133 ostensibly normal adult volunteers indicated that calcitonin was undetectable in 54% of the samples (<10 ng/L) and ranged from 11 to 200 ng/L in the remaining 46% of the sera. A more detailed description of these data will be presented in a forthcoming communication where we identify the major immunochemical forms of calcitonin in sera and thyroids. Goat antiserum to rabbit gamma globulin was provided by Dr. P. Aldenderfer (Biological Markers Laboratory) and used at a final dilution of 130-fold.

Results

Figure 1 shows the isoelectric focusing pattern (electrophorogram) of nonradioactive synthetic human calcitonin. The major peak of immunoreactive calcitonin exhibited an apparent isoelectric point of 7.9. Minor contaminants in the synthetic peptide preparation with isoelectric points of 6.4 and 8.6 were detected.

In the early experiments, we used QUSO-G32 to remove free iodine and [125I]iodolactoperoxidase before electrofocusing of the reaction mixtures. Radiodination of human calcitonin by use of Chloramine T resulted in a heterogeneous population of [125I]iodopeptides that exhibited more acidic isoelectric points than calcitonin itself and whose incidence varied between reactions (Figure 2A, B). Although producing fewer [125I]iodopeptides, radiodination of human calcitonin by use of lactoperoxidase also yielded acidic peptides that varied between reactions (Figure 2C, D). The examples in Figure 2 represent the extremes in products obtained from both radiodination procedures.

Table 1 lists the molar ratios of reactants used in several laboratories (8, 10–12) for the Chloramine T radiodination of human calcitonin. It is apparent that although reaction times are relatively short (15 to 30 s), the ratios of oxidizing and reducing agents vary greatly. For comparison with the results from our procedures, human calcitonin was radiodinated by procedure 2 (8), adsorbed to and eluted from QUSO-G32, and electrofocused. The results (Figure 3) demonstrate that the products of the established radiodination procedure for human calcitonin are heterogeneous and that the isoelectric points are similar to those obtained with our procedure. The disadvantage to the established procedure was...
Fig. 2. Electrophoretograms of human calcitonin after radioiodination with Chloramine T (A and B) and lactoperoxidase (C and D) for 2 min and 10 min, respectively. Reactions were extracted with QUSO-G32. Columns were run for 66–68 h at 4–7 °C and 1 W of power.

the low rate of iodine incorporation (<20%) which, without further purification, would decrease assay sensitivity. With our procedure, iodine incorporation into calcitonin exceeds 80%; however, decreasing the reaction time decreases the incorporation of label.

Biphasic, irreproducible calcitonin dose–response curves (Figure 4) were observed in the radioimmunoassay when we used QUSO-G32 extracted [125I]iodopeptides from three lactoperoxidase reactions (iodinations no. 21, 41, and 54). Furthermore, we observed that serial dilutions of sera from
Table 1. Molar Ratios of Reactants in the Chloramine T Iodination of Human Calcitonin

<table>
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<th>Procedure no.</th>
<th>Reference</th>
<th>Calcitonin</th>
<th>Iodine</th>
<th>Chloramine T</th>
<th>Sodium metaiodobenzoate</th>
<th>Time, s</th>
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</thead>
<tbody>
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<td>1</td>
<td>Current</td>
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<td>1.0</td>
<td>15.8</td>
<td>11.9</td>
<td>120</td>
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<tr>
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<td>60.7</td>
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</tr>
<tr>
<td>3</td>
<td>(10)</td>
<td>1</td>
<td>0.37</td>
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<td>2140.0</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>(11)</td>
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<td>1124.0</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>(12)*</td>
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<td>1.47</td>
<td>4370.0</td>
<td>434 900.0</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>(12)</td>
<td>1</td>
<td>1.47</td>
<td>21 400.0</td>
<td>4300.0</td>
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</tr>
</tbody>
</table>

* Dr. C. W. Cooper, University of North Carolina School of Medicine, Chapel Hill, NC, personal communication.

* NA = Not available in the original communication.

patients with medullary carcinoma of the thyroid characteristically produced dose–response curves that did not parallel the entire standard calcitonin dose–response curve.

Alternative purification procedures were investigated, in an attempt to decrease the heterogeneity of the [\(^{125}\)I]iodo-peptides. Anion-exchange chromatography on QAE-Sephadex, with use of a linear gradient of 0–0.3 mol/L sodium chloride in 0.1 mol/L tris(hydroxymethyl)aminomethane, pH 10.5, effectively fractionated the radioiodination reaction mixtures (Figure 5). Nonradioactive calcitonin eluted before, but contiguous with, the first peak of [\(^{125}\)I]iodopeptides.

A second peak of radioactivity from the QAE resin coelutes with free iodine and [\(^{125}\)I]iodolactoperoxidase and therefore was not further examined. Application of 1 mol/L sodium chloride failed to remove additional radioactivity, although some was still bound at the top of the resin bed (5–10% of total radioactivity applied to column). The \([^{125}\)I] labeled peptides eluted from the column in the first peak of radioactivity, generally 40–50% of the total radioactivity because of the self iodination by the enzyme, were electrofocused (Figure 6).

Highly purified preparations of \([^{125}\)I] labeled human calcitonin were obtained from the QAE resin after radioiodination by

Fig. 3. Electrophoretogram of human calcitonin after its radioiodination with Chloramine T for 30 s

Fig. 4. Standard human calcitonin dose–response curves in the radioimmunoassay

The designs, \(\text{lg}_{-54}, \text{lg}_{-41}, \text{lg}_{-21}\), denote the iodination identification number. The mean ± SD (n = 6) is indicated for the calcitonin dose–response curve obtained with iodination number \(\text{lg}_{-54}\). The serum sample was from a patient with medullary carcinomas of the thyroid and the dilution curve (Δ–Δ) obtained by using iodination number \(\text{lg}_{-41}\)

Fig. 5. Purification of lactoperoxidase-iodinated human calcitonin on QAE-Sephadex (4 × 1 cm)

A 0–0.3 mol/L sodium chloride gradient in 0.1 mol/L tris(hydroxymethyl)aminomethane, pH 10.5, was used for elution. Illustrated are the elution profiles for human calcitonin (•–•), \([^{125}\)I]iodo-lactoperoxidase (O–O), and free iodine plus \([^{125}\)I]iodolactoperoxidase (O–•–O)
Chloramine T (Figure 6A, B) and lactoperoxidase (Figure 6C, D). The major products obtained from the Chloramine T reaction exhibited isoelectric points of pH 6.8 to 7.0; with lactoperoxidase they were pH 7.3 to 7.4.

The $^{125}$I-labeled human calcitonins obtained from the electrophoretograms (Figures 2 and 6) were digested with pronase and the labeled amino acid was identified by thin-layer chromatography. $^{[125]}$Diiodohistidine was identified as the labeled amino acid in the $^{125}$I-labeled human calcitonin, with isoelectric points of pH 7.9 and 8.0; $^{[125]}$moniodohistidine was associated with the $^{125}$I-labeled peptide with an isoelectric point of 5.3. $^{[125]}$Diiodotyrosine was associated...
with the $^{125}$I-labeled peptides exhibiting isoelectric points of 6.8, 7.0, 7.3, and 7.4.

For lactoperoxidase-radioiodinated human calcitonin from QAE, calcitonin dose–response curves for mixtures that contained normal human plasma and charcoal-treated normal human plasma were typically both linear and parallel with serial dilutions of plasma from patients with medullary carcinoma of the thyroid (Figure 7). These data are in marked contrast to the data obtained with the unpurified $^{125}$I-labeled peptides (Figure 4). In addition, more than 85% of the QAE-derived $^{125}$I-labeled peptide was precipitated in the presence of excess rabbit antiserum to human calcitonin (500-fold dilution).

**Discussion**

The intent of this communication is to illustrate a method for examining radiolabeled peptides rather than to prove that one method of radioiodination is superior to another for the labeling of a specific peptide.

Results of the present investigation indicate that: (a) examination of small quantities of highly radioactive peptides was accomplished by isoelectric focusing, (b) alteration of the peptide was detected following radioiodination by both Chloramine T (regardless of reaction time) and lactoperoxidase, and (c) characterization of labeled peptides by identification of the labeled amino acid provides little information on the basic structural integrity of the labeled peptide.

The data in this report question what the “optimum” conditions are for the radioiodination of a peptide. Generally, radioiodination conditions are optimized either with respect to retaining biological activity or to labeling a peptide to high specific activity for radioimmunoassay.

When the goal is the former then it is essential for the investigator to demonstrate that the purification procedure used is capable of completely separating labeled from nonlabeled peptide, so that the results of the bioassay are specific to the labeled peptide. Frequently, because of the stringent structural requirements and the presence of amino acids that are sensitive to destruction by oxidation and reduction conditions (cystine, methionine, and tryptophan), the radioiodinated form of the peptide has little biological activity. Regardless of biological activity, the labeled peptide should be examined and defined by some physical/chemical method so that it can be reproduced by other investigators.

If instead the peptide is to be labeled to high specific activity for radioimmunoassay, it is important that the immunological determinant for the antiserum is not altered by the radioiodination conditions. In this communication on human calcitonin, the site required for biological activity (13) is equivalent to the immunological determinant for the antiserum. Therefore, our work on labeled calcitonin provides insight into the loss of biological activity of radioiodinated human calcitonin, as reported by Scarpace and Defos (14). The method chosen to examine radioiodinated calcitonin was isoelectric focusing, which is rapid, convenient, and applicable to peptides such as calcitonin that have few ionizing amino acid side-chains. Radioiodination of calcitonin, involving only the substitution of an iodine atom(s) on the ring structure of tyrosine or histidine, or both, results in a relatively small decrease in the isoelectric point of the labeled peptide when compared to calcitonin. However, a dramatic shift in the isoelectric point would indicate that the amino terminal disulfide bond essential for biological activity had been oxidized or reduced by the radioiodination conditions. Oxidation of the disulfide bond to cysteic acid (pK$a$ of 1.3) would decrease the isoelectric point of the peptide. Electrofocusing of QUSO-extracted radioiodination reactions demonstrates the presence of a small amount of labeled peptide with an isoelectric point of less than 3.0 (Figure 2), suggesting oxidation of the disulfide bond. This peptide was only minimally detectable when the radioiodination reaction was purified by anion-exchange chromatography (Figure 6). The two negatively charged cysteic acid residues have a high binding affinity for the anion-exchange resin, and the oxidized peptide would be eluted subsequent to a peptide with an intact disulfide bond. Reduction of the disulfide bond to the thiol (pK$a$ of 10.5) would greatly increase the isoelectric point of the peptide, and we did not observe labeled peptides with isoelectric points greater than calcitonin in any of the electrophoretograms. Radioiodination conditions sufficient to oxidize the disulfide bond would also oxidize methionine (also essential for biological activity) to the sulfoxide and sulfone. However, peptides containing oxidized methionine would not be distinguished by electrofocusing because there would be no change in the net charge of the peptide.

The data indicating oxidation of the disulfide bond are supported by the observations on the human calcitonin dose–response curves obtained with QUSO-extracted (Figure 4) and QAE-purified (Figure 7) $^{125}$I-labeled peptides. The non-linear calcitonin dose–response curves obtained when using the QUSO-extracted $^{125}$I-labeled peptide demonstrate that, with some of the labeled peptides, alteration to the amino terminal immunological determinant site for the antiserum had occurred. Conversely, the linear calcitonin dose–response curves indicate that the disulfide bond was unaltered in the QAE-purified labeled peptides. The antiserum used in this communication is, to our knowledge, the only one specific to the easily oxidized disulfide bond of calcitonin, which explains why the nonlinear calcitonin dose–response curves have not been reported previously. Collectively, the presence of a labeled peptide with an isoelectric point of less than 3.0 and the nonlinear calcitonin dose–response curves associated with the QUSO-extracted labeled peptides help to explain the loss of biological activity of calcitonin (14) radioiodinated with 20-fold more Chloramine T than we used in this report (see procedure 4, Table 1).

It is frequently assumed that the length of the radioiodination reaction is the major factor in promoting diiodo-substitution and damage to the peptide. The reports from other laboratories and our data suggest that the amino acid composition and the tertiary structure of the peptide are equally
important. Krohn et al. (15) reported that radioiodination of a number of peptides by the same Chloramine T procedure resulted in a variety of monoiodo- and diiodo-substituted tyrosine and histidine residues. The proportion of each halogenated amino acid varied among the peptides, indicating that the structure of each peptide influenced the site and degree of iodine substitution more than did the radioiodination procedure. The report of Bayse et al. (16) illustrated that the amino acids adjacent to tyrosine dramatically affected the rate of tyrosine iodination. Roche et al. (17) reported that the rates of monoiodo- and diiodo-substituted tyrosine and histidine formation were independent. Our data indicate that while minor amounts of [125I]monooiodohistidine- and [125I]-
diiodohistidine-containing calcitonin were obtained, the principal products of both Chloramine T (isoelectric point of 6.8) and lactoperoxidase-directed (isoelectric point of 7.36) radioiodinations contain [125I]diiodotyrosine. Furthermore, it can be concluded on the basis of the higher isoelectric point for the major product that the lactoperoxidase procedure produces less structural alteration to human calcitonin during radioiodination than does the Chloramine T procedure. The reaction time used for the Chloramine T radioiodination of human calcitonin is apparently of limited importance, because the products obtained from a 30-s reaction (Figure 3) were analogous to the products obtained from a 2-min reaction (Figure 2). In addition, the [125I]diiodotyrosine-containing peptide eluted from the anion-exchange resin has the desired maximum specific activity of 4550 Ci/mmol for use in the radioimmunoassay.

In summary, our results demonstrate that for human calcitonin we have optimized the radioiodination and purification procedures to obtain a 125I-labeled peptide that retains the structural integrity of the biologically important amino terminal disulfide bond.

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References