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Appendix: Reversible Modification of Cysteine with Cyanogen Bromide

Abstract

When a peptide containing cysteine, specifically the dipeptide L-cysteinyl-L-cysteine, is exposed to 0.2 M cyanogen bromide in 70% formic acid, the cysteine is quantitatively modified to products that will not react with iodoacetic acid. When these products are exposed to dithiothreitol, the cysteine is quantitatively regenerated and reacts quantitatively with iodoacetic acid to yield the carboxymethylated product. The ability of a cyanogen bromide fragment to incorporate iodoacetic acid only after exposure to a thiol such as dithiothreitol cannot be used as a criterion for the presence of a disulfide in the native protein.

Introduction

A criterion that is often used to define an intramolecular cystine within a single intact peptide is the ability of that peptide to incorporate an alkylating agent such as iodoacetic acid only after exposure to a thiol such as 2-mercaptoethanol or dithiothreitol. There are, however, modifications of cysteine other than participation in a cystine that can be reversed nucleophilically by treatment with a thiol and would be mistakenly assigned as participation in a cystine by this criterion. In particular, when the parent polypeptide has been cleaved with cyanogen bromide in 70% formic acid, a free cysteine in the original protein could become cyanylated, formylated, or turned into the sulfinic acid of cysteine. It is also possible for adventitious cystines to form under these conditions. Any of these modifications could be reversed by dithiothreitol.

Kao and Karlin (1986) isolated two cyanogen bromide fragments from the α polypeptide of acetylcholine receptor that comprised amino acids 118–144 and 179–207 in the amino acid sequence of this polypeptide. Both of these cyanogen bromide fragments incorporated ^{14}C from iodo[^{14}C]acetamide, but only after exposure to dithiothreitol. By this criterion they concluded that the native protein contained intramolecular cystines within these regions of its amino acid sequence.

The experiments described here, however, demonstrate that free cysteine, upon exposure to cyanogen bromide in 70% formic acid, is quantitatively and covalently modified to several products. These products cannot be alkylated by iodoacetic acid directly, but after exposure to dithiothreitol they covalently incorporate iodoacetic acid to yield quantitatively the carboxymethyl derivatives of the original cysteines.

Experimental Procedures

Materials. Iodoacetic acid was purchased from Aldrich Chem. Co.; tris(hydroxymethyl)aminomethane (Tris), from Fisher Chemical Co.; and cysteine, from Calbiochem Corp. Cyanogen bromide was purchased from Matheson, Coleman, and Bell, and the transparent, colorless crystals of the sublimed solid attached to the walls of the container were used. The bis dipeptide, L-cystinyl-L-cystine, was purchased from Research Plus Inc. The grams per mole of the solid bis dipeptide were calculated from the elemental analysis provided by the manufacturer. The initial solution of L-cystinyl-L-cystine prepared for the experiment was 4.58 mg dissolved in 1.00 mL of 30 mM Tris-HCl, pH 9.0.

Chromatography. Samples were prepared for chromatography by removing 20 μL from the experimental solution and adding it to the appropriate amount (0.5–1.0 mL) of a mixture of equal parts of 0.5 mM norleucine in 0.01 M HCl and 0.066 M sodium citrate adjusted to pH 2.2 with hydrochloric acid. The samples were run on a Beckman 118C amino acid analyzer using a 3-h, single column program (Beckman Instruments, 1975). The peptides eluting from the chromatographic column (0.6 × 34 cm) were detected and quantified by the standard, continuous-flow, postcolumn ninhydrin system associated with the instrument.

Results

The bis dipeptide L-cystinyl-L-cystine was dissolved at 10 mM in a solution of 30 mM Tris-HCl, pH 9.0, that had been sparged with argon. Dithiothreitol was added as the solid to a final concentration of 50 mM, and the solution was sealed under argon for 30 min at room temperature. A sample (calculated to be 100 nmol of amino termini) was then removed and submitted to chromatographic analysis on a Model 118C amino acid analyzer (Beckman Corp.). A peak emerged with a retention time (68 min) between those of norleucine (63 min) and tyrosine (73 min) (retention time is the difference between the time at which a retarded component is recorded on the chromatogram and the time at which an unretarded component is recorded on the chromatogram). If it is assumed that this peak represents L-cysteinyl-L-cysteine, its yield of ninhydrin color is only 2% that of glycine at 570 nm. Cysteine itself has a yield of ninhydrin color that is only 8% that of glycine. This fact and the fact that peptides typically produce colored products in lower yields than do amino acids after reaction with ninhydrin are consistent with the assignment of this peak as L-cysteinyl-L-cysteine.

A sample of the reduced dipeptide (calculated to be 10 μmol of amino termini) was removed, and solid iodoacetic acid was added to a final concentration of 200 mM. The pH was raised to 9, and the reaction was allowed to proceed for 30 min at room temperature. A portion of this sample (calculated to be 100 nmol of amino termini) was submitted to chromatographic analysis. The peak previously assigned to L-cysteinyl-L-cysteine was completely absent (<20%), and a new peak, not present on the previous analysis, appeared. Its retention time (13 min) was intermediate between those of serine (9 min) and glutamate (14 min). If it is assumed that the carboxymethylation was quantitative and that this peak represents *S*,*S*′-bis(carboxymethyl)-L-cysteinyl-L-cysteine, then its yield of ninhydrin color is 65% that of glycine. A similar increase in the yield of ninhydrin color is observed when cysteine (8% that of glycine) is converted to *S*-(carboxymethyl)cysteine

(60% that of glycine). The significant decrease in retention time is consistent with the addition of two negative charges to the dipeptide due to carboxymethylation.

A portion (calculated to be 8 μmol of amino termini) of the remaining reduced, unalkylated L-cysteinyl-L-cysteine was submitted to lyophilization and dissolved to a final concentration of 8 mM in 0.2 M cyanogen bromide dissolved in 70% formic acid. The reaction was allowed to proceed for 24 h at 4 °C. The mixture was submitted to lyophilization to remove the cyanogen bromide, the product was redissolved to a nominal concentration of 20 mM in amino termini, and the pH was adjusted to 9, by using the Tris remaining in the sample as a buffer. A portion of this material (calculated to be 200 nmol of amino termini) was submitted to chromatographic analysis. Four new peaks were observed whose retention times (84, 89, 97, and 101 min) were later than the retention time assigned to L-cysteinyl-L-cysteine (68 min) and later than the change to the third buffer of the chromatographic regime (82 min) but earlier than the retention time of ammonia (105 min). Their total yield of ninhydrin color (12% that of glycine) was also greater than that of L-cysteinyl-L-cysteine (2% that of glycine). They are presumed to be products of oxidation, formylation, or cyanylation of L-cysteinyl-L-cysteine brought about by the exposure of the dipeptide to cyanogen bromide and formic acid. No L-cysteinyl-L-cysteine (<10%) remained following the treatment.

A sample of the L-cysteinyl-L-cysteine (approximately 3 μmol of amino termini in 0.15 mL) that had been exposed to cyanogen bromide and formic acid was removed. It was flushed with argon, and dithiothreitol (7.5 μmol) was added to a final concentration of 50 mM. It was sealed under argon, and the reaction was allowed to proceed for 30 min at room temperature. Solid iodoacetic acid (30 μmol) was then added, the pH was adjusted to 8, and the reaction was allowed to proceed for 30 min at room temperature. A portion of this sample (calculated to be 180 nmol of amino termini) was submitted to chromatographic analysis, and a high yield (180 nmol) of *S*,*S*′-bis(carboxymethyl)-L-cysteinyl-L-cysteine was observed. The three products produced in the highest yield by the modification of L-cysteinyl-L-cysteine by cyanogen bromide and formic acid, those with retention times of 84, 89, and 101 min, had disappeared (<2% remaining) during the treatment with dithiothreitol and iodoacetic acid.

If the treatment with dithiothreitol was omitted and only the treatment with iodoacetic acid was performed, no *S*,*S*′-bis(carboxymethyl)-L-cysteinyl-L-cysteine (<1.0 nmol) was produced, and no loss of the products of modification by cyanogen bromide and formic acid was observed. The fact that less than 1 nmol of *S*,*S*′-bis(carboxymethyl)-L-cysteinyl-L-cysteine was produced when treatment with dithiothreitol was omitted means that less than 1% of the L-cysteinyl-L-cysteine survived modification by cyanogen bromide and formic acid.

Discussion

These results demonstate that the exposure of the dipeptide L-cysteinyl-L-cysteine, and by extention any peptide containing two adjacent cysteines or one or more separated cysteines, to 0.2 M cyanogen bromide in 70% formic acid produces derivatives of cysteine in overall quantitative yield. A high percentage of these derivatives (>95%) yield *S*-(carboxymethyl)cysteine upon reaction with iodoacetic acid, but only if they have been first exposed to dithiothreitol or presumably any other thiol. As this is the criterion used by Kao and Karlin (1986) to identify cystines in cyanogen bromide fragments, the cysteines designated by them to be cystines could have been cysteines in the native protein rather than cystines. This criterion cannot distinguish cysteines from cystines.

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