Identification and Location of \( \varepsilon \)-N-Trimethyllysine in Yeast Cytochromes c*

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SUMMARY

Iso-I cytochrome c of Saccharomyces cerevisiae contains 1 residue of \( \varepsilon \)-N-trimethyllysine at position 72 in the sequence. Iso-II cytochrome c from this species and cytochrome c from Candida krusei also contain 1 residue of this amino acid. Thus, cytochromes c of Ascomycetes contain 1 residue and, as previously reported, a higher plant contains 2 residues of \( \varepsilon \)-N-trimethyllysine. This amino acid is completely absent from a large variety of animal cytochromes and from other types of proteins.

In an earlier publication (1), we reported that Neurospora cytochrome c and wheat germ cytochrome c contain 1 and 2 residues of \( \varepsilon \)-N-trimethyllysine, respectively. The locations of this unusual amino acid in the amino acid sequences of these proteins were also identified. Further, it was noted (1) that \( \varepsilon \)-N-trimethyllysine was absent from cytochromes c of various animal species previously studied in this laboratory.

In order to determine further the distribution and significance of \( \varepsilon \)-N-trimethyllysine we now wish to report studies on a variety of additional cytochromes c and on several other proteins.

EXPERIMENTAL PROCEDURES

Materials

Saccharomyces cerevisiae (baker's yeast) iso-I and iso-II cytochromes c, as well as those from Pacific lamprey, Samia cynthia (a moth), bullfrog, snapping turtle, and turkey, and alfalfa ferredoxin, were all generous gifts from Dr. Emanuel Margoliash. Candida krusei cytochrome c was purchased from the Sankyo Company, Ltd., Tokyo, Japan. Subtilisin Carlsberg was obtained from Novo Industri A/S, Copenhagen. Collagen (bovine Achilles tendon) and protamine were purchased from Mann and Paul-Lewis Laboratories, respectively. \( \varepsilon \)-N-Monomethyllysine, \( \varepsilon \)-N-dimethyllysine, and \( \varepsilon \)-N-trimethyllysine were obtained as previously described (1). All reagents were of analytical grade.

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Methods

Amino Acid Analyses—Amino acid analyses for methylated lysines were performed as follows. Proteins and peptides were hydrolyzed for 20 to 24 hours in 6 N HCl at 110°, under vacuum. HCl was removed over NaOH in a heated vacuum desiccator at 45°. Aliquots representing at least 0.1 pmole of each protein were analyzed on a Spinco automatic amino acid analyzer, model 120B, by the procedure described previously (1). In addition to these analyses, performed on Spinco resin type UR-30, analyses were also performed on a Durrum resin, type DC-2, which gave considerably improved resolution under the same conditions of elution (see Fig. 1).

Chymotryptic Hydrolysis—Iso-I cytochrome c of baker's yeast (70 mg, approximately 5 pmoles), dissolved in 10 ml of water, was hydrolyzed with 0.7 mg of chymotrypsin at 38° and pH 8.0. The solution was maintained at pH 8.0 with 0.05 N NaOH, and the course of the reaction was followed by using a Radiometer pH-stat. Hydrolysis was terminated by the addition of acetic acid to pH 3.0 and the acidified digest was degassed and applied directly to a column of Dowex 50-X2 (see below).

Column Chromatography of Chymotryptic Peptides—A column (1.9 × 150 cm) of Dowex 50-X2 (200 to 400 mesh), previously equilibrated at 45° with 0.2 M pyridine-acetic acid at pH 3.1, was used. The molarity refers to the pyridine concentration. Elution was initiated with 2320 ml of the 0.2 M pyridine-acetic acid buffer at pH 3.1, and 10-ml fractions were collected. A linear gradient was set up between 3 liters of 0.2 M pyridine-acetic acid at pH 3.1 and 3 liters of 2 M pyridine-acetic acid at pH 5.0. This gradient was followed by another 1 liter of 4 M pyridine-acetic acid at pH 5.0. A 0.5-ml aliquot from alternate tubes was analyzed by the ninhydrin method after alkaline hydrolysis (2). The elution profile is shown in Fig. 2. The material in each peak was analyzed after acid hydrolysis for the presence of methylated lysines. \( \varepsilon \)-N-Trimethyllysine was located only in the material from the peak indicated by a solid bar in Fig. 2. Methylated lysines were not detected in any other peptide fraction of the chymotryptic hydrolysate.

Purification of \( \varepsilon \)-N-Trimethyllysine-containing Chymotryptic Peptide—The material obtained from the fraction containing \( \varepsilon \)-N-trimethyllysine (Fig. 2) was resolved by descending paper chromatography in butanol-1:pyridine-acetic acid-water (15:10: 3:12, by volume). The peptide containing \( \varepsilon \)-N-trimethyllysine (Peptide C-TML) was then separated from a contaminating...
Fig. 1. Separation of $\epsilon$-N-methyllysines on a column (0.9 x 48 cm) of Durrum resin type DC-2, eluted at a flow rate of 28.2 ml per hour with 0.35 M sodium citrate buffer, pH 5.84, at 28°. Different amounts of the various amino acids were used. Acidic and neutral amino acids elute before 1 hour; arginine elutes at 18 hours.

Fig. 2. Fractionation of chymotryptic peptides from Saccharomyces cerevisiae iso-I cytochrome c on a column (1.9 x 150 cm) of Dowex 50. See the text for details. The fraction containing a peptide with $\epsilon$-N-trimethyllysine as a constituent is shown by the solid bar.

TABLE I

| Amino acid compositions of yeast cytochromes c | Saccharomyces cerevisiae | Candida bruxii |
|---------------------------------------------|-------------------------|----------------|
| Iso I                                       | Iso II                  |
| Tyrosine                                    | 5.05 (5)                | 5.08 (5)       | 5.09 (5)       |
| Phenylalanine                               | 4.00 (4)                | 4.85 (5)       | 3.90 (4)       |
| Lysine                                      | 16.0 (16)               | 17.8 (18)      | 11.8 (12)      |
| $\epsilon$-N-Trimethyllysine               | 1.07 (1)                | 1.08 (1)       | 1.16 (1)       |
| Histidine                                   | 3.72 (4)                | 4.08 (4)       | 3.76 (4)       |
| Arginine                                    | 2.56 (3)                | 2.62 (3)       | 3.56 (4)       |

RESULTS

As reported in the previous communication (1), $\epsilon$-N-trimethyllysine, synthesized from $\alpha$-acetyllysine and methyl iodide, was characterized by its migration in several electrophoretic and chromatographic systems. We have now compared the properties of our synthetic product to those of an authentic sample of $\epsilon$-N-trimethyllysine, kindly supplied by Dr. N. L. Benoiton. Both samples of $\epsilon$-N-trimethyllysine eluted in the same position from a column of Durrum resin type DC-2 (see Fig. 1) with the pH 5.84 buffer system (1, 3), and emerged as a single peak when mixed together. The two samples also migrated in identical fashion on electrophoresis at pH 1.9 on Whatman No. 3MM paper.

Digestion with Carboxypeptidases A and B—Peptide C-TML (0.025 pmole) was hydrolyzed with 20 μg each of carboxypeptidases A and B in 0.1 M NH₄HCO₃ at pH 8.0 and 40° for 20 hours.

Expressed in moles per mole of peptide. Analysis of a hydrolysate of the peptide by the usual analyzer buffer systems showed 2 residues of lysine with a shoulder on the trailing edge. The presence of equal amounts of lysine and $\epsilon$-N-trimethyllysine was shown by analysis of a hydrolysate eluted with the pH 5.84 buffer.

Only lysine (no $\epsilon$-N-trimethyllysine) and tyrosine were present as determined by analysis with the pH 5.84 buffer system and by electrophoresis at pH 1.9.

as on paper chromatography with butanol-1-acetic acid-water (200:30:75), with butanol-1-pyridine-acetic acid-water (15:10:3:12), and with the phenol-cresol system (1, 4). A single ninhydrin-positive spot was obtained when a mixture of both samples was run in each of these systems.

We express appreciation to Professor N. L. Benoiton for sending a description of the characterization of his sample of $\epsilon$-N-trimethyllysine, prior to publication (J. H. Seely and N. L. Benoiton, manuscript in preparation). The characterization included melting point determinations and elemental analysis of the chloroaurate salt (C₃₁H₄₂N₄O₁₃·AuCl₄).

TABLE II

| Composition | Carboxy- | Carboxy- |
|-------------|---------|---------|
| Peptidase   | Peptidase |
| A (1 hr)    | A + B (20 hr) |
| Aspartic acid | 1.25 (1) |          |
| Threonine    | 1.04 (1) |          |
| Proline      | 0.90 (1) |          |
| Leucine      | 0.91 (1) |          |
| Tyrosine     | 0.91 (1) |          |
| Lysine       | 0.96 (1) | 0.95 (1) |
| $\epsilon$-N-Trimethyllysine | 1.04 (1) | 1.05 (1) |

* Expressed in moles per mole of peptide. Analysis of a hydrolysate of the peptide by the usual analyzer buffer systems showed 2 residues of lysine with a shoulder on the trailing edge. The presence of equal amounts of lysine and $\epsilon$-N-trimethyllysine was shown by analysis of a hydrolysate eluted with the pH 5.84 buffer.

* Tyrosine was the only residue released by carboxypeptidase A as judged by examination of the reaction mixture by electrophoresis at pH 1.9 and by paper chromatography with butanol-1-glacial acetic acid-water (200:30:75, by volume).

* Only lysine (no $\epsilon$-N-trimethyllysine) and tyrosine were present as determined by analysis with the pH 5.84 buffer system and by electrophoresis at pH 1.9.
Anino acid analyses of *S. cerevisiae* iso-I and iso-II cytochromes c and of *C. kruasei* cytochrome c revealed the presence of 1 residue of ε-N-trimethyllysylne in each of these proteins (Table I). ε-N-Monomethyllysine and ε-N-dimethyllysine were not present in any of the hydrolysates of these proteins. No methylated lysines were detected in any of the other five cytochromes c or the additional proteins examined.

The location of the ε-N-trimethyllysyl residue was determined only in yeast iso-I cytochrome c. The methylated lysine was found exclusively in Peptide C-TML (Table II). The composition of this peptide corresponds to residues 68 through 75, according to the usual numbering for mammalian cytochromes c (9), and corresponds to residues 73 through 79, Leu-Thr-Asp-Pro-Lys-Lys-Tyr, in the numbering system for the sequence of baker's yeast cytochrome c as reported by Narita and Titani (6).

In the mammalian numbering system, the position of the trimethylated lysine was determined to be at residue 72, as shown by the release of tyrosine (residue 74) and unmodified lysine (residue 73) upon treatment with carboxypeptidases A and B, respectively (Table II). Liberation of residue 72 by carboxypeptidase B would not be expected, since it is adjacent to a prolyl residue. Thus, the location of the modified residue corresponds exactly to that previously determined for the ε-N-trimethyllysyl residue in the cytochromes c of *Neurospora* and of 1 of the 2 residues of ε-N-trimethyllysine in wheat germ cytochrome c (1).

**DISCUSSION**

With the present and previous (1) findings it is evident that ε-N-trimethyllysine is absent from animal cytochromes. We have been able to detect none of the methylated lysine derivatives in this protein derived from any of the following representative species of the various classes of vertebrates, e.g. lamprey, dogfish, bullfrog, turtle, rattlesnake, turkey, or various mammals, or from the insect, *S. cynthia*. In contrast, the cytochromes c of the Ascomycetes—*Neurospora crassa*, *S. cerevisiae* (iso-I and iso-II forms), and *C. kruasei*—contain a single residue of ε-N-trimethyllysine and it is located at residue 72 in *Neurospora* and iso-I of *Saccharomyces*, according to the numbering of the mammalian cytochromes, and, presumably, at the same site in the others. Although the sequences of the iso-I cytochrome c of *Saccharomyces* (6) and of *C. kruasei* cytochrome c (5) have been reported, the presence of ε-N-trimethyllysine was overlooked, presumably because of its elution with lysine on the usual analytical system for amino acids.

Wheat germ cytochrome c contains 2 residues of this amino acid, one also being at residue 72 and the other at residue 86 (1). Investigations on the composition and sequences of other higher plant cytochromes c by Boulter and his associates (7) have now shown that all such cytochromes likewise contain 2 residues of ε-N-trimethyllysine and that these are present at the same sites.

From these studies there appears to have been a sharp evolutionary differentiation in the development of specific methylation enzymes in *Ascomycetes* and higher plants. The work of Scott and Mitchell (8) on *Neurospora* has shown that methylation occurs after the complete cytochrome c is formed; i.e. in early cultures, cytochrome c is present in unmethylated form and is subsequently modified. Although plants thus contain systems for methylation of cytochrome c, and possibly other proteins, and the free amino acid, ε-N-trimethyllysine, has been found in plant tissues (9, 10), there clearly is considerable specificity involved in this methylation. First, this amino acid and other methylated lysines are not present in all plant proteins, e.g. squash seed globulin (11), alfalfa ferredoxin, and pea seedling histone IV (11), although the corresponding histone of bovine thymus contains mono- and dimethyllysine (12–14). Second, the sites of trimethylation in the fungal and plant cytochromes are highly specific.

Dickerson and coworkers (9) have deduced a three-dimensional structure for equine cytochrome c which has permitted identification of many of the amino acid side chains. From their work there is a crevice at the front of the molecule. In addition, "there appears to be a hole or channel leading from the back of the molecule to the heme. The side chains of lysyl residues 72 and 86 are on the exterior of the molecule, and are located near the hole or channel." Thus, residues 72 and 86 may represent an important interaction site with another component of the mitochondrion, possibly the cytochrome oxidase (1).

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