The Post-translational Modifications of the Nuclear Encoded Subunits of Complex I from Bovine Heart Mitochondria*

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Bovine complex I is an assembly of 46 different proteins. Seven of them are encoded in mitochondrial DNA, and the rest are nuclear gene products that are imported into the organelle. Fourteen of the nuclear encoded subunits have modified N termini. Many of these post-translational modifications have been deduced previously from intact protein masses. These assignments have been verified by mass spectrometric analysis of peptides. Thirteen of them are N-α-acetylated, and a 14th, subunit B18, is N-α-myristoylated. Subunit B18 forms part of the membrane arm of the complex, and the myristoyl group may attach subunit B18 to the membrane. One subunit, B12, has a particularly complex pattern of post-translational modification that has not been analyzed before. It is a mixture of the N-α-acetylated form and the form with a free N terminus. In addition, it has one, two, or three methyl groups attached to histidine residues at positions 4, 6, and 8 in various combinations. The predominant form is methylated on residues 4 and 6. There is no evidence for the methylation of histidine 2. Subunit B12 is also part of the membrane arm of complex I, and it probably spans the membrane once, but as its orientation is not known, the methylation sites could be in either the matrix or the intermembrane space. These experiments represent another significant step toward establishing the precise chemical composition of mammalian complex I. Molecular & Cellular Proteomics 4:693–699, 2005.

Complex I (NADH:ubiquinone oxidoreductase) in mammalian mitochondria is one of the most complicated enzymes yet characterized. It is a membrane-bound multisubunit complex with a mass of about 1 MDa (1). Its structure is L-shaped with one arm lying in the plane of the inner membrane and the other extending into the mitochondrial matrix (2). Complex I contributes to the conversion of redox energy into the proton motive force by coupling electron transfer from NADH to coenzyme Q to extrusion of protons from the mitochondrial matrix into the intermembrane space. It is thought that trans-

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and its subcomplexes by fractionation on 1D or 2D gels (5, 7). On a 1D gel subunit B14 co-migrated with B14.5a. Other subunits were obtained by reverse phase HPLC on a column of Aquapore RP-300 (2.1 × 100 mm; PerkinElmer Life Sciences) (5, 7) or, in the case of subunit B14.5a, on a PRP-3 column (2.1 × 150 mm; Hamilton Co., Reno, NV) in 0.1% trifluoroacetic acid. Proteins were eluted from the PRP-3 column with a gradient of solvent containing 0.05% trifluoroacetic acid, 40% acetonitrile, and 50% propan-2-ol at a flow rate of 0.1 ml/min. The details of the gradient were as follows: 5–15 min, 0–20% solvent; 15–75 min, 20–80% solvent; 75–85 min, 80–100% solvent. The eluate was monitored at 225 nm. Subunit B13 was obtained by HPLC fractionation of subcomplex I on Aquapore RP-300 (7) followed by 1D SDS-PAGE of a selected fraction.

**Digestion of Subunits of Complex I**—Proteins in bands and spots excised from gels were digested by “in-gel” cleavage (17) at 37 °C with trypsin, chymotrypsin, or endoproteinase Lys-C in buffer consisting of 20 mM Tris-HCl, pH 8.0, and 5 mM calcium chloride, with endoproteinase Asp-N in 20 mM Tris-HCl, pH 8.0, with endoproteinase Arg-C in 24 mM Tris-HCl, pH 8.0, containing 4 mM calcium chloride, 5 mM dithiothreitol, and 0.4 mM EDTA or at room temperature with cyanogen bromide (18). The proteolytic enzymes were purchased from Roche Diagnostics GmbH. Samples of N-terminally modified subunits that had been purified by HPLC were dried and redissolved in 25–100 mM ammonium bicarbonate containing either trypsin (0.3 ng/µl plus 0.4 µM calcium chloride), chymotrypsin (5 ng/µl), or endoproteinase Lys-C (8 ng/µl) and incubated at 37 °C. Digested samples were either stored at −20 °C or acidified with formic acid (0.1–1.0% (v/v) final concentration) before analysis.

**Mass Spectrometry**—Peptide digests of subunits of complex I were analyzed in a MALDI-TOF mass spectrometer (ToFSpec 2E spectrometer, Micromass, Altrincham, UK) using α-cyano-4-hydroxycinnamic acid as the matrix. The instrument was calibrated either with bovine trypsin autolysis products (m/z values, 2163.057 and 2273.16) and a calcium-related matrix ion (m/z value, 1060.048) (5) or with added peptide standards (renin substrate, angiotensin I, and adrenocorticotropic hormone fragment 18–39 human with pro-

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### Table I

**Sources of N-terminally modified subunits of bovine complex I**

| Subunit | Source | Purification |
|---------|--------|--------------|
| B22     | lβ     | 1D gel       |
| B18     | lβ     | 1D gel       |
| B17,2   | lα     | 1D gel       |
| B17     | lβ     | 1D gel       |
| B16,6   | lα     | 1D gel       |
| B15     | lγ     | 1D gel       |
| B14,7   | lγ     | 1D gel       |
| B14.5a  | lα     | HPLC         |
| B14.5b  | lα     | 1D gel       |
| B14     | lα     | 1D gel       |
| B13     | lα     | HPLC and 1D gel |
| B12     | lβ     | HPLC or 1D gel |
| B9      | Cl     | 2D gel       |
| B8      | lα     | HPLC         |

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### Table II

**Mass spectrometric analysis of N-terminally modified peptides from subunits of complex I from bovine heart mitochondria**

In the “peptide” column, the letters T, R, M, D, Y, and K indicate peptides obtained by cleavages with trypsin, endoproteinase Arg-C, cyanogen bromide, endoproteinase Asp-N, chymotrypsin, and endoproteinase Lys-C, respectively. Sequences deduced from the data in the supplemental data section are underlined. Ac- and Myr- denote acetyl- and myristoyl- respectively.

| Subunit | Peptide | Residues | m/z (z+) | Mass (MH+) |
|---------|---------|----------|----------|-----------|
|         |         |          | Observed | Calculated |
| B22     | T1      | 1–14     | 797.01 (2)| 1593.02 | 1592.81 |
| B18     | T1      | 1–7      | 417.83 (2)| 834.66 | 834.56 |
| B17,2   | T1–2    | 1–9      | 594.44 (2)| 1187.88 | 1171.69 |
| B17     | R1      | 1–9      | 611.18 (2)| 1221.36 | 1221.61 |
| B16,6   | M1      | 1–10     | 486.40 (2)| 971.80 | 971.52 |
| B15     | R1      | 1–9      | 563.68 (2)| 1126.55 | 1126.65 |
| B14,7   | D1      | 1–9      | 603.70 (2)| 1206.40 | 1206.66 |
| B14.5a  | Y1–2    | 1–9      | 561.26 (2)| 1121.52 | 1121.57 |
| B14.5b  | Y1      | 1–11     | 649.56 (2)| 1298.12 | 1297.61 |
| B14     | D1      | 1–22     | 743.91 (3)| 2229.23 | 2230.23 |
| B13     | T1–2    | 1–6      | 336.27 (2)| 671.54 | 671.45 |
| B9      | K1      | 1–9      | 523.70 (2)| 1046.40 | 1046.60 |
| B8      | K1      | 1–12     | 592.05 (2)| 1183.10 | 1182.71 |
tonated molecular masses of 1296.6853, 1758.9331, and 2465.1989 Da, respectively). With the exception of subunit B12, peptide sequences were obtained by tandem MS in an ESI-Q-TOF instrument (Micromass) as described previously (5). Some digests were introduced by nanoelectrospray; others were prefractionated by liquid chromatography “on-line” to the mass spectrometer (see supplemental data). Samples to be analyzed by nanoelectrospray were desalted first on a ZipTipC18 pipette tip (Millipore) and eluted in 50% acetonitrile containing 1% formic acid. The N-terminal peptides from the tryptic digest of subunit B12 were desalted by hydrophilic interaction chromatography on ZipTipHPL. They were eluted in 50% acetonitrile containing 10 mM formic acid and sequenced by tandem MS following CID with argon in a MALDI-Q-TOF instrument (Micromass) using \( \alpha \)-cyano-4-hydroxy-trans-cinnamic acid as the matrix. The instrument was calibrated with the same standards used for calibrating the ESI-Q-TOF instrument (5).

RESULTS AND DISCUSSION

Modifications at the N Terminus of Subunits—From earlier analyses of intact subunits by Edman sequencing, it was known that 14 of the nuclear encoded subunits of bovine complex I (listed in Table I) have modified N-terminals (4, 7, 13, 19). By analyzing various enzyme digests and also cyanogen bromide digests of the subunits by MALDI-TOF mass spectrometry, peptides representing the N-terminal regions of these subunits were identified. From them, N-terminal peptides that were considered to be suitable for mass spectrometric sequencing were selected (see Table II). Then these selected peptides were sequenced by tandem mass spectrometry (see Table II and supplemental data). In the majority of cases (eight of 14), the sequences of the N-terminal peptides were deduced from the C terminus up to residue 2. The masses of residue 1 plus the modifying group and the independent knowledge of the N-terminal residue from the sequences of the subunits allowed the modifying group to be identified (see supplemental data). In one additional case, the mass of the N-terminal dipeptide plus the modifying group in another case, the N-terminal tripeptide plus the modifying group allowed the modifying groups to be identified in a similar way. The N-terminal region of subunit B14 was particularly difficult to sequence, and the identification of its modifying group depended on the mass of the N-terminal octapeptide and independent knowledge of the sequence of the subunit in this region. With the exception of subunit B18, which is modified by an N-terminal myristoyl moiety, the other 13 subunits were \( \alpha \)-acetylated. Molecular mass measure-
ments showed that the N-terminal residues of subunits B14.5b and B12 were modified partially (4, 12). This observation was verified for subunit B12 as described below and for subunit B14.5b by analysis of peptides by MALDI-TOF MS (data not shown) and by analysis of the acetylated version by tandem MS (see supplemental data).

**Methylation of Subunit B12**—The partial acetylation of subunit B12 was confirmed by the MALDI-TOF spectrum of the tryptic digest of subunit B12 (Fig. 1). The spectrum is dominated by peaks at 1278.608 and 1293.06, 1307.05, 1321.07, and 1335.10. Ions arising by internal fragmentation are denoted by . Ions arising from isobaric sequences are denoted by (see Table III). For explanations of the origins of these fragments and interpretation of the tandem MS data, see the supplemental data section and Supplemental Tables S3–S8.

**Fig. 2.** Analysis of modified peptides from the N-terminal region of subunit B12 of bovine complex I. Fragment ions were produced by CID of singly positively charged N-terminal tryptic peptides of subunit B12 in a MALDI Q-TOF mass spectrometer (see Table III). A–F, fragment ion spectra of ions with m/z values of 1265.03, 1279.05, 1293.06, 1307.05, 1321.07, and 1335.10. Ions arising by internal fragmentation are denoted by . Ions arising from isobaric sequences are denoted by (see Table III). For explanations of the origins of these fragments and interpretation of the tandem MS data, see the supplemental data section and Supplemental Tables S3–S8.
data are summarized in Table III. The spectra and their interpretations are shown in Fig. 2, and additional details of interpretation are given in the supplementary data.

These data show that the most abundant methylation pattern is for histidines 4 and 6 to be modified together. There is also evidence for methylation of histidine 4 and histidine 6 separately and of histidines 4, 6, and 8 together, but these species are much less abundant than the form methylated on residues 4 and 6. Both N-α-acetylated and non-acetylated versions of all of these methylation patterns were observed. There was no evidence for other possible patterns of methylation of histidines 4, 6, and 8 or for methylation of histidine 2 alone or in any combination with histidines 4, 6, and 8. Subunit B12 has two additional histidines at C-terminal residues 76 and 77. Neither of them is modified (see Supplemental Table S2).

Histidine residues could possibly be methylated on either the N-1 or N-3 nitrogen of the imidazole ring. At present, there is no information about which of these two sites is modified in the methylated histidines in subunit B12.

**Biological Significance of the Post-translational Modifications in Complex I**—The N-α-acetylation of eukaryotic proteins is one of the most common post-translational modifications, and more than 50%, and possibly as high as 80–90%, of mammalian intracellular proteins are modified in this way (20, 21). The modification takes place co-translationally after
the emergence of 20–30 amino acids of the nascent polypeptide from the ribosome. Often, but not always, it involves the removal of the initiator methionine followed by N-acetylation of residue 2; 78% (11 of 14) of the modified subunits of complex I are acetylated in this manner.

Among the complex I subunits investigated here, the initiator methionine is acetylated in subunits B14.5b and B17.2, whereas it has been removed, and residue 2 has been acetylated in all the other subunits that were investigated, excepting subunit B18 (see below). In general, glycine, alanine, serine, methionine, and aspartic acid are dominant as the N-terminal residues of N-α-acetylated proteins (20). In bovine complex I, acetylated N-terminal residues are alanine in nine subunits, serine in two subunits, and methionine in two further subunits (see Table II). The 14th modified subunit, B18, is myristoylated on glycine 2 (4). It has been noted previously that myristoylation occurs only on N-terminal glycine residues after removal of the initiator methionine and that small and uncharged amino acids are preferred at residues 2 and 5 (22). The N-terminal residue of subunit B18 is glycine, and alanine residues are found at positions 2 and 5, and so the sequence conforms to the canonical myristoylation motif. Why the various subunits of complex I need to be N-terminally acetylated is unclear, but it may be related to the post-translational transfer of the proteins into mitochondria. The biological significance of myristoylation is also obscure. Subunit B18 is a component of subcomplex Iβ, representing part of the membrane arm of complex I, and the myristoyl group may be responsible for binding the subunit to the inner mitochondrial membrane.

Methylation of the side chains of basic amino acids is also a common post-translational modification, but methylation of histidine residues is much rarer than methylation of arginine and lysine residues (23). Among the best known examples are the methylation of histidines to produce 3-methylhistidine in muscle function, although muscle breakdown associated with fasting and athletic activity is monitored by measuring 3-methylhistidine in urine (27). The nickel enzyme methyl-coenzyme M reductase, which catalyzes the final step in methane synthesis, contains a 1-methylhistidine residue (28). The biological significance of the methylation of subunit B12 of complex I is obscure. It is not known at what stage of the synthesis and assembly of the subunit into complex I the modification takes place. This subunit is part of the membrane arm of the complex, and it probably spans the membrane once (4, 11). The locations of the N and C termini have not been established, and so it is not known on which side of the inner mitochondrial membrane the methylation sites lie. In contrast to N-α-acetylation, which appears to be a permanent modification, protein methylation is reversible by demethylases, and methylation-demethylation reactions can have a regulatory function (29). This aspect of complex I remains to be investigated.

The nuclear encoded subunits of bovine complex I contain other significant post-translational modifications that have not been discussed in this study. Around six to eight iron-sulfur clusters are incorporated into four or five other subunits (11), and one additional subunit is an acyl carrier protein carrying a phosphopantethenic acid residue on a serine residue (30). One subunit of the complex with a molecular mass of 10,566 Da has resisted all attempts to sequence it, but its N terminus appears to be modified (7).
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