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IDENTIFICATION OF THE INITIALLY NBD-LABELED ESSENTIAL TYROSINE RESIDUE IN BOVINE HEART MF_1-ATPASE

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SUMMARY: Bovine heart MF_1-ATPase was labeled with limiting amounts of [^{14}C]NBD-Cl([^{14}C]4-chloro-7-nitro-2,1,3-benzoxadiazole) and the resulting radioactive label on the essential Tyr was stabilized by reduction with zinc in the presence of multidentate ligand EDTA and redox mediator 4,4'-dipyridyl. Subsequent treatment of the labeled protein with cyanogen bromide and separation of the reaction mixture by ion-exchange chromatography yielded essentially only one radioactive polypeptide. Further cleavage of this polypeptide with TPCK-trypsin, lactonization of the terminal homoserine residue and reaction with derivatized polystyrene resin gave a shorter peptide attached to the solid support which contained all the radioactivity. Edman degradation showed that the amino acid sequence of this peptide was Glu·Gly·Asn·Asp·Leu·Tyr·His·Glu·Met, which corresponds to residues 192-200 in the beta subunit of bovine heart MF_1-ATPase as determined by Runswick and Walker (1983). Since this specifically labeled Tyr-197 is separated by only one amino acid residue from the essential Glu-199 which was labeled specifically with dicyclohexylcarbodiimide by Yoshida et al. (1982) it seems most likely that both Tyr-197 and Glu-199 play direct roles in the catalytic hydrolysis and synthesis of ATP.

The single essential tyrosine residue in MF_1-ATPase which can be selectively labeled by 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) is of great interest because of its possible role in catalysis (1,2). But identification of this essential Tyr has been hampered by the lability of this NBD-label which is readily removed even by relatively weak nucleophiles. A simple method has been developed in this work to stabilize the [^{14}C]NBD-label on the essential Tyr of MF_1 by reduction with zinc in the presence of EDTA as sequestering agent and 4,4'-dipyridyl (Methyl Viologen) as redox mediator. The resulting reduced label, presumably [^{14}C]4-[7-amino-2,1,3-benzoxadiazolyl]-, is sufficiently stable to survive cyanogen bromide treatment, chromatographic separations and attachment of the isolated radioactive peptide to solid support for sequence determination.

MATERIALS AND METHODS

Materials. MF_1-ATPase was prepared by the method of Knowles & Penefsky (3). The enzyme with specific activities in the range 60-100 units/mg was stored at 4°C as precipitate in a buffered medium containing 0.25 M sucrose, 50 mM Tris-

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HCl buffer at pH 8.0, 2 mM EDTA, 4 mM ATP and 2 M $(NH_4)_2SO_4$. The [^{14}C]NBD-Cl (109 Ci/mol) was purchased from Research Products International Corporation. Enzymes and biochemicals for ATPase assay were obtained from Sigma Chemical Company. Reagents and specially purified solvents for the determination of amino acid sequence were purchased from Pierce Chemical Company.

Labeling of MF_1-ATPase and Stabilization of the Label by Reduction. In a typical experiment, 1.9 mL of MF_1-ATPase suspension (56 mg) were centrifuged and the pellets were redissolved in 3.0 mL of a pH 7.5 buffer containing 50 mM Hepes-NaOH + 0.2 M sucrose + 2 mM EDTA + 5 mM ATP. The solution was centrifuge-eluted through a Sephadex G-50-80 column which had been pre-equilibrated with the same buffer to remove ammonia and Tris. An acetone solution (80 µL) containing 0.18 µmol of [^{14}C]NBD-Cl (20 µCi) was evaporated to dryness with N_2. The residue was immediately allowed to react with the above MF_1-ATPase solution at room temperature in the dark for 2 hrs. The resulting solution was then mixed with 1.8 mL of 0.1 M EDTA solution (pH 7.0). The mixture was subsequently flushed with N_2, and 5 mg of Methyl Viologen (MV) plus some granular zinc were added. The reduction of labeled MF_1 was allowed to proceed at room temperature in the dark with intermittent shaking for about 30 min until the dark purplish blue color persisted after standing without shaking. Then 8 mg of solid sodium dithionite ($Na_2S_2O_4$) were added, and after 10 more min the supernatant was put in dialysis tubes and dialyzed against 3 L of dilute Hepes-NaOH buffer (pH 7.5) containing EDTA for 38 hrs with 4 changes of bath solution. The dialyzed solution (6 mL) contained 47.8 mg of labeled MF_1 with a Label/MF_1 molar ratio of 0.79. The protein concentration was determined by the Coomassie Blue adsorption method (5). Radioactivity was assayed by liquid scintillation counting.

Cleavage of Labeled Protein and Separation of Polypeptides. The labeled protein (43 mg) was cleaved by reacting with 3 times its weight of CNBr in 0.1 N HCl solution for 24 hrs at room temperature. The resulting mixture was lyophilized and the polypeptides were redissolved in 2 mL of 20 mM ethanolamine-HCl buffer at pH 9.5. The solution was injected onto a Mono Q anion exchange column (Pharmacia) and eluted by the same buffer with a linear NaCl gradient. The pooled radioactive fraction (indicated by the horizontal bar in Fig. 2) was lyophilized, redissolved in 1 mL of 0.1% trifluoroacetic acid (TFA) and applied to a HPLC column (Radial Pak C_{18} Cartridge, 8 mm x 10 cm, Waters Associates), which had been equilibrated with 100% CH_3OH for 20 min and pre-eluted with 0.1% TFA (Fig. 3A). The pooled radioactive fractions (indicated by the horizontal bar in Fig. 3A) were again lyophilized and then digested for 16 hrs at 23°C with TPCK—trypsin (Sigma) in 1 mL of 1% NH_4HCO_3 solution at an estimated enzyme/substrate molar ratio of 1/100. The products of tryptic digestion were subsequently again applied onto the C_{18}-column and eluted with 0.1% TFA (Fig. 3B).

Determination of Sequence. The final radioactive fraction collected from the C_{18}-column contained about 30% of the initial total radioactivity. This was lyophilized and then coupled to triethylenetetramine (TETA) polystyrene resin by the homoserine lactone method (6). The coupled peptide was sequenced by manual Edman degradation according to a previously described procedure (7) in a miniature glass column, using acetone, methanol and dichloroethane as the wash solvents. The product from each cycle was converted to the corresponding phenylthiohydantoin by treatment with concentrated HCl for 10 min at 80°C and subsequently analyzed by HPLC with a C_{18}-column (3.9 mm x 30 cm, Waters Associates).

Amino Acid Analysis. The radioactive polypeptide coupled to TETA-polystyrene resin was hydrolyzed in 6 N HCl for 22 hrs at 110°C. The resulting amino acids were derivatized with o-phthalaldehyde and analyzed by HPLC in a reverse phase C_8-column (Merck Hibar) with a 3-stage elution procedure (8).

RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectrum of O-NBD-MF_1 with a maximum at 385 nm as reported previously (1). Spectrum A was obtained from a 18.9 µM

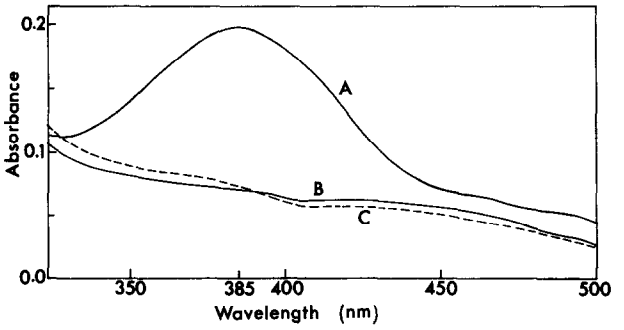

Figure 1. Absorption spectra of covalently labeled MF_1-ATPase at different reaction stages. (A) O-NBD-MF_1; (B) O-ABD-MF_1; (C) O-ABD-MF_1 further treated with $Na_2S_2O_4$. The concentration of labeled MF_1 was 18.9 μM, with a label/MF_1 molar ratio of 0.77 ± 0.01.

O-NBD-MF_1 solution with a label/MF_1 molar ratio of 0.78. After reduction with Zn + MV + EDTA and elution-centrifugation through Sephadex G-50-80, the filtrate gave Spectrum B when the absorbance was adjusted to the same protein concentration. The complete disappearance of absorption peak at 385 nm after reduction suggests the successful conversion of the 7-nitro group of the radioactive label to an amino group. The enzyme with reduced label, presumably O-[7-amino-2,1,3-benzoxadiazolyl]MF_1 or O-ABD-MF_1, was found to have a label/MF_1 molar ratio of 0.75. Subsequent treatment of O-ABD-MF_1 with sodium dithionite and elution-centrifugation through Sephadex G-50-80 produced a filtrate with absorption spectrum C (adjusted to the same protein concentration) in Fig. 1 with a label/MF_1 molar ratio of 0.77.

Since the labeled MF_1 samples with Spectra A,B & C respectively have essentially the same label/MF_1 molar ratio, we may conclude that complete reduction of the 7-nitro group by Zn + MV + EDTA can take place without cleaving the labile covalent bond between the O-NBD-label and the Tyr of MF_1, and that the resulting O-ABD-label on MF_1 can survive subsequent exposure to sodium dithionite in solution. Direct reduction of the O-NBD-label by dithionite in alkaline solution was found to cleave the covalent bond between the label and MF_1. The similarity of Spectra B & C suggests that the treatment of O-ABD-MF_1 with sodium dithionite may be unnecessary.

The separation of polypeptides obtained by CNBr-cleavage of O-ABD-MF_1 is shown in Fig. 2. Essentially only one radioactive polypeptide fraction (indi-

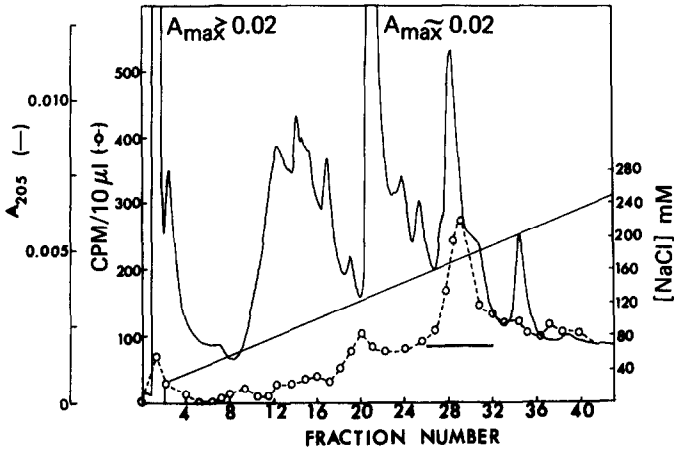

Figure 2. Isolation of radioactive peptides from the cyanogen bromide digest of [^{14}C]O-ABD-MF$_1$ by Mono Q anion exchanger column. The column was equilibrated with 20 mM ethanolamine-HCl buffer at pH 9.5. The linear gradient of NaCl in buffer was generated at a flow rate of 1 mL/min. The UV absorbance was monitored at 205 nm with a Gilson Holochrome. The pooled fractions used for sequence analysis, indicated by the horizontal bar, contained 36% of the total radioactivity applied onto the column.

cated by high A_{205} and cpm) was eluted from the Mono Q anion exchanger column. It contained 36% of the total radioactivity put on the column. Fig. 3A shows that subsequent fractionation of this radioactive polypeptide sample by HPLC with C_{18}-column did not lead to substantial further purification.

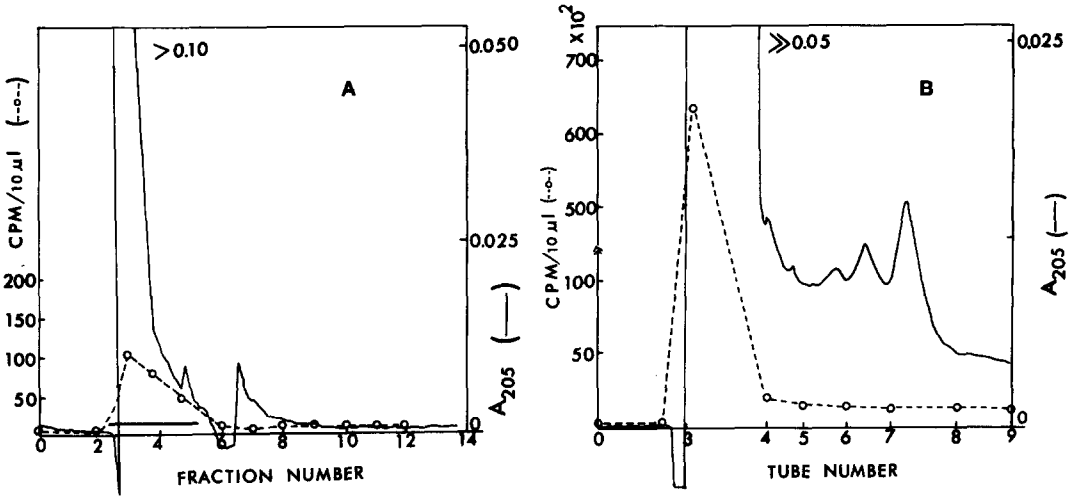

Figure 3. Further purification of the radioactive peptides by HPLC. (A) The pooled radioactive fractions from Mono Q column were applied to Radial Pak C_{18} column (8 mm x 10 cm, Waters Associates) and eluted with 0.1% TFA as described under Materials and Methods. (B) Final purification of the radioactive peptides with Radial Pak C_{18} column after cleavage with TPCK-trypsin. Each peak was collected separately and assayed for radioactivity.

The radioactive fraction eluted from C_{18}-column was further cleaved with TPCK-trypsin. The resulting mixture was again fractionated with C_{18}-column (Fig. 3B) and a fraction which contained 30% of the total initial radioactivity was collected. This radioactive fraction was then treated with CF_3COOH to lactonize the terminal homoserine residue and subsequently allowed to react with TETA-polystyrene resin. It was found that essentially all the radioactivity became attached to the resin column and that the eluate plus washings of the column contained only 3% of the total radioactivity.

Edman degradation of the resin-attached radioactive peptide and subsequent separation of the resulting PTH-amino acids by HPLC gave the results in Table I. After each reaction cycle, only one A_{254} peak was found by HPLC which was identified by the subsequent injection and elution of a standard sample of the suspected PTH-amino acid. The degradation ended at the 9th cycle when the homoserine derived from methionine was reached. The data in Table I show unambiguously that the amino acid sequence of the radioactive peptide attached to

Table I. Identification of PTH-amino acids obtained by Edman degradation of the radioactive peptide attached to TETA-polystyrene resin by HPLC[a]

Reaction Cycle	Eluent[b]	Elution time (min)	
		Sample	Standard
1	A	4.4	4.6 (PTH-Glu)
2	A	7.4	7.3 (PTH-Gly)
3	A	5.2	5.5 (PTH-Asn)
4	A	3.6	3.4 (PTH-Asp)
5	B	6.2	6.1 (PTH-Leu)
6	B	4.5	4.5 (PTH-Tyr)
7	B	16.5	16.5 (PTH-His)
8	A	4.3	4.6 (PTH-Glu)

[a]Complete hydrolysis of this peptide gave the following number of moles of the amino acid per mole of leucine: Asn + Asp, 2.3; Glu, 2.2; Gly, 1.08; His, 0.81; Leu, 1; Tyr, 1.1.

[b]Composition of eluents: (A) 12 mM KH_2PO_4-NaOH buffer, pH 7.0, in 12% (v/v) CH_3OH; (B) 12 mM KH_2PO_4-NaOH buffer, pH 7.0, in 40% (v/v) CH_3OH. The phenylthiohydantoins PTH-Leu, PTH-Tyr and PTH-His were eluted too slowly by the first solvent, hence a second mobile phase was used.

polystyrene resin was Glu·Gly·Asn·Asp·Leu·Tyr·His·Glu, which corresponds to the sequence of residues 192-199 in the beta-subunit of bovine heart MF_1-ATPase (6). The [^{14}C]ABD-label was not sufficiently stable to survive more than three Edman degradation cycles. However, because of the spectral evidence (1) and the presence of only one Tyr in this peptide which carried most of the radioactivity, we conclude that the label was attached to Tyr-197. Since this specifically labeled Tyr-197 is separated by only one amino acid residue from the essential Glu-199 which was also specifically labeled by dicyclohexylcarbodiimide (9), it seems most likely that both Tyr-197 and Glu-199 play direct roles in the catalytic hydrolysis and synthesis of ATP.

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