Articles

Subunit II of the Cytochrome bo_3 Ubiquinol Oxidase from *Escherichia coli* Is a Lipoprotein

Jixiang Ma,‡ Andromachi Katsonouri, and Robert B. Gennis*

School of Chemical Sciences, 600 South Mathews Street, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: The purified *Escherichia coli* cytochrome bo_3 ubiquinol oxidase contains four subunits that are each integral components of the cytoplasmic membrane. The molecular weight of each of the subunits has been determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI). The observed molecular weight of subunit II (CyoA) is considerably less than the calculated value from the deduced amino acid sequence, indicating possible posttranslational processing. The similarity of a portion of the sequence near the N-terminus of CyoA with the sequences of known prokaryotic membrane-bound lipoproteins suggested that CyoA is proteolytically processed to generate an N-terminus at Cys25, and that Cys25 is covalently modified by the addition of lipids. This would be consistent with the observed molecular mass, and was confirmed by demonstrating the incorporation of radioactive palmitic acid into subunit II of the cytochrome bo_3 oxidase. Site-directed mutagenesis replacing Cys25 by alanine prevents the processing, generating a precursor form of CyoA with a higher molecular mass. The C25A mutant of CyoA still assembles as an active quinol oxidase capable of supporting growth of the cells by aerobic respiration. Hence, this unusual processing of a cytoplasmic membrane protein, which is already anchored to the membrane by two transmembrane helices, is not essential for either assembly or function.

The cytochrome bo_3 ubiquinol oxidase, located in the cytoplasmic membrane of *Escherichia coli*, catalyzes the two-electron oxidation of ubiquinol-8 and the four-electron reduction of molecular oxygen to water (Anraku & Gennis, 1987; Trumpower & Gennis, 1994). This enzyme is a member of the superfamily of proton-pumping heme−copper terminal oxidases which includes the mitochondrial cytochrome *c* oxidase (Calhoun et al., 1994; García-Horsman et al., 1994; Gennis, 1993; Saraste, 1990). The free energy resulting from the net electron transfer reaction is conserved in the form of a proton electrochemical gradient across the cytoplamic membrane. The purified oxidase contains four subunits by SDS−PAGE analysis (Minghetti et al., 1992; Tsubaki et al., 1993).

The genes encoding the cytochrome bo_3 oxidase have been cloned and sequenced (Au & Gennis, 1987; Chepuri et al., 1990), and the operon containing the structural genes of cytochrome bo_3 oxidase has been shown to contain five open reading frames, *cyo*A, *cyo*B, *cyo*C, *cyo*D, and *cyo*E (Chepuri et al., 1990). The first four ORFs encode subunits II, I, III, and IV, respectively (Minghetti et al., 1992), whereas the *cyo*E encodes the protoheme IX farnesyltransferase (heme O synthase) (Saiki et al., 1992, 1993). Hydropathy profile analysis of the amino acid sequences of subunits I, II, III, and IV indicates that all of the subunits contain transmembrane helical spans (Chepuri et al., 1990), and the X-ray structures of the related cytochrome *c* oxidases have confirmed the predicted number of membrane spans for subunits I, II, and III. As a rule, the intrinsic proteins of the *E. coli* cytoplasmic membrane are not synthesized as precursors with cleavable N-terminal signal sequences. It is shown in the current work that CyoA (subunit II) is an exception. CyoA is initially synthesized with an N-terminal signal sequence which is processed to form the mature subunit II, which is a lipoprotein with covalently attached lipids at the new N-terminus. The data suggest that, after processing, the N-terminus is Cys25. Cys25 appears to be modified to form glycerylcysteine (*S*-[propane-2′,3′-diol]-3-thioaminopropionic acid), to which two fatty acid residues are linked by two ester linkages, and one fatty acid residue is probably attached by an amide linkage, similar to that reported for the major outer membrane lipoprotein of *E. coli* (Braun, 1975). Site-directed mutagenesis experiments are reported that block the processing, but do not prevent the assembly of a functional quinol oxidase.

MATERIALS AND METHODS

Materials. The restriction enzymes *Kpn*I, *Sal*I, and *Msp*I were purchased from Bethesda Research Laborataries. The restriction enzyme *Hha*I, T4 DNA ligase, and T4 DNA kinase were purchased from New England Biolabs. T4 DNA polymerase, exonuclease III, and Sequenase kits were obtained from U.S. Biochemical Corp. The nucleotides dATP, dGTP, dCTP, dTTP, and 5-Me-dCTP were from Pharmacia LKB Biotechnology, Inc. ATP and phenylmethanesulfonyl fluoride (PMSF) were from Sigma. Oligonucleotides used to generate mutants or as sequencing primers were obtained from the Biotechnology Center at the

* Corresponding author. Telephone: 217-333-9075. Fax: 217-244-3186. Email: Gennis@Aries.SCS.UIUC.edu.

‡ Present address: Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Science, Research Triangle Park, NC 27709.

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Table 1: Primers Used for Mutagenesis and Their Corresponding Amino Acid Changes

Mutant	Mutating primer	Amino Acid Sequence
WT		MRLRKYNKSLGWLSLFAGTVLLSGCNSALL
R2L/R4L	5'GGTCGTTAAATG**CT**ACTC**CT**GAAATACAAT3'	M**LL**LKYNKSLGWLSLFAGTVLLSGCNSALL
K5N/K8N	5'AGACTCAGGAA**T**TACAATAA**T**AGTTTG3'	MRLR**N**YNN**S**LGWLSLFAGTVLLSGCNSALL
R4S/K5N/K8N	5'ATGAGACTCAG**T**AA**T**TACAATAA**T**AGTTT3'	MRL**S**N**S**YNN**S**LGWLSLFAGTVLLSGCNSALL
R2S/R4S/K5N/K8N	5'TTAAATGAG**T**CTCAG**T**AA**T**TACAATAA**T**AGTTT3'	M**S**L**S**N**S**YNN**S**LGWLSLFAGTVLLSGCNSALL
R2L/R4L/K5I/K8I	5'GGTCGTTAAATG**CT**ACTC**CT**GA**T**ATACAATA**T**AAGTTTGGGA3'	M**LLLI**YNI**S**LGWLSLFAGTVLLSGCNSALL
C25A	5'CTCAGTGGC**GC**TAATTCTGCG3'	MRLRKYNKSLGWLSLFAGTVLLSG**A**NSALL
ΔR2S9	5'AATTGAGGTCGTTAAATGTTGGGATGGTTGTCATTA3'	M--------LGWLSLFAGTVLLSGCNSALL

University of Illinois (Urbana, IL). Goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate and prestained SDS−PAGE standards were purchased from Bio-Rad Laboratories. ^{14}C-Labeled palmitate (50−60 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. Ni-NTA resin was from QIAGEN, Inc.

Electroelution of Peptides following SDS−PAGE and Sample Preparation for Mass Spectrometry. Preparative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out using the system of Laemmli (1970). The separating gel contained 12.5% acrylamide. Purified cytochrome *bo*$_3$ (4 mg/mL) was incubated with an equal volume of 2× SDS gel-loading buffer at room temperature for 30 min before being loaded onto the gel. After electrophoresis, the gel was rapidly rinsed with distilled water, and then stained with freshly prepared 0.1% Coomassie blue R-250 in 10% methanol, 0.5% acetic acid for 30 min. The destaining was performed in 10% methanol. The four bands corresponding to subunits I, II, III, and IV, respectively, were cut out with a razor blade. The gel slices were soaked in 1% SDS for 30 min, and then equilibrated in a 1:10 dilution of the electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). Electroelution was performed using the electroelutor/concentrator kit obtained from ISCO, Inc. by following the manufacturer's instructions. The collected samples were each concentrated to about 60 μL by using Centricon units (Amicon), and then diluted with 2 mL of water containing 0.25% SDS. The samples were again concentrated to about 40−60 μL, and the procedure of dilution and concentration was repeated once again. In order to remove SDS, the concentrated samples (about 120 μL each) were placed in 1.5-mL Eppendorf tubes and heated at 100 °C for 75 s. After cooling, 4 volumes of acetone were added to each of the samples, and they were mixed well using a vortexer. The mixtures were then placed in a −20 °C freezer for at least 30 min. The pellets were collected by centrifugation at 4 °C for 10 min, and then washed with a cold mixture of 80% acetone and 20% of a solution containing methanol/water/acetic acid in the volume ratio 50/49/1. Finally, the dried pellets were dissolved in a minimal volume of formic acid.

Mass Spectrometry. Molecular mass determination of electroeluted peptides was performed using a laser desorption VG TofSpec "time-of-flight" matrix-assisted laser desorption/ionization mass spectrometer (MALDI). This was done in the Mass Spectrometry Laboratory of the University of Illinois. The procedures to apply the mass spectrometry technique to membrane proteins are described elsewhere (Ghaim et al., 1997).

Radioisotope Labeling. The strain GO105/pJRHisA was used for all of the labeling studies (Rumbley et al., 1997). The host strain GO105 is Δ*cyd cyo* and, hence, does not express either of the two *E. coli* terminal oxidases. Plasmid pJRHisA contains the wild-type *cyo* operon, encoding cytochrome *bo*$_3$ with six consecutive histidine residues attached to the C-terminus of subunit II (Rumbley et al., 1997). One milliliter of overnight LB culture was inoculated into 50 mL of M63 minimal medium supplemented with 0.3% lactate, 0.3% casamino acids, and 0.5% glycerol. Ampicillin (100 μg/mL) and kanamycin (50 μg/mL) were also included in the growth medium. After the cells grew with shaking at 37 °C for about 6 h, [1-^{14}C]palmitic acid was added to the medium to give a final concentration of 5 μCi/mL. The labeling was continued for about 50 min, and then the cells were harvested.

Purification of Cytochrome bo$_3$. Harvested cells were washed once with 10 mM Tris (pH 8.0), and then resuspended in 1 mL of 50 mM glucose, 10 mM EDTA, and 25 mM Tris (pH 8.0) containing 4 mg/mL lysozyme. Lysozyme was added just prior to use. After incubation at room temperature for 5 min, the resulting spheroplasts were pelleted by centrifugation at 4 °C for 10 min, and resuspended in 400 μL of 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM $MgSO_4$, 0.5 mM PMSF, and 1 μM DNase I. Then 50 μL of 10% Triton X-100 and 50 μL of 12.5% octyl glucoside were added to the solution. The sample was left on ice for 30 min with occasional stirring. Cell debris were removed by centrifugation, at which point the sample was diluted by the addition of 800 μL of 50 mM potassium phosphate (pH 8.0). Then 30 μL of Ni-NTA resin which was equilibrated with the phosphate buffer was added to sequester the solubilized cytochrome *bo*$_3$. The resin was washed 4 times with a solution containing 50 mM potassium phosphate, 500 mM NaCl, 10% glycerol, and 0.1% Triton X-100, pH 8.0. The enzyme was released from the washed resin by adding to the resin 100 μL of 500 mM imidazole, 0.1% Triton X-100, and 50 mM potassium phosphate (pH 8.0). To this was added 100 μL of 2× SDS gel-loading buffer, and, after incubation at room temperature for 30 min, the sample was examined by SDS−PAGE. After fixing (7% acetic acid, 25 % methanol), the gel was dried under vacuum and then exposed to an intensifying screen for 4 days. The image was obtained by scanning the screen with a Molecular Dynamics PhosphImager, and the data were processed with the software ImageQuant (both from Molecular Dynamics Inc.).

Mutagenesis. Table 1 shows the list of oligonucleotides and the corresponding changes used in this work. The site-directed mutagenesis was performed as previously described (Vandeyar et al., 1988). The single-stranded DNA template used for mutagenesis was an M13 derivative, M13SK, which was constructed by cloning the *Sal*I−*Kpn*I fragment from

the *cyo* operon into M13mp18. This fragment contains the N-terminus of subunit II plus the promoter region. After the desired replacements were verified on the M13 phage by DNA sequencing using the USB Sequenase kit protocol, the *Sal*I−*Kpn*I fragment was cloned back into the *cyo* operon. The mutations were confirmed by DNA sequencing.

Cell Growth and Membrane Preparation. GO105 cells harboring wild-type or mutated plasmids were grown aerobically as previously described (Lemieux et al., 1992). Cells were washed once with 10 mM Tris (pH 8.0) and passed twice through a French pressure cell at 1200 psi. The solution was centrifuged at 9000*g* for 30 min to remove cell debris, and the supernatant was collected. The cytoplasmic membranes were collected by centrifugation at 45 000 rpm in a Ti60 rotor for 1.5 h, and then homogenized in 50 mM potassium phosphate (pH 7.5) for subsequent characterization.

Western Immunoblotting Analysis. A previously described procedure was used for Western immunoblotting analysis (Harlow & Lane, 1988) following SDS−PAGE with a 12% polyacrylamide gel. Proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories) at 100 V for 1 h. Rabbit polyclonal antibodies were raised against purified cytochrome bo_3, and the components directed against subunit I and subunit II were strip-purified. The anti-subunit I and anti-subunit II antibodies were combined and used for Western immunoblotting. The antibodies were obtained initially in the University of Illinois Hybridoma Laboratory.

Miscellaneous. Optical spectroscopy was performed with a DW2000 UV−Vis spectrometer (SLM Instruments, Inc.) Cytochrome bo_3 was purified as previously described (Minghetti et al., 1992). Ellman's test for determining the number of reactive cysteine residues in the protein was performed as previously described (Ellman, 1959; Riddles et al., 1979). Oxidase activity was determined as previously described (Ma et al., 1993). In order to determine whether or not physiologically functional oxidases were expressed in each of the mutants, a genetic complementation test was conducted following a previously published procedure (Lemieux et al., 1992) using the host strain GO105, which does not support aerobic growth on nonfermentable substrates.

RESULTS

Cytochrome bo_3 from *E. coli* was prepared using ion-exchange chromatography as previously described (Minghetti et al., 1992). The four subunits, with apparent molecular masses of (I) 55, (II) 31, (III) 20, and (IV) 11 kDa are shown in Figure 1. Apparent molecular masses of the four subunits of the oxidase observed in the SDS−PAGE analysis were 55, 31, 20, and 11 kDa, respectively. Each of the four subunits was removed from the gel by electroelution, and its molecular mass was obtained by MALDI analysis (Ghaim et al., 1997). The observed values of the masses along with those calculated based on the DNA sequences are presented in Table 2. Only subunit II (CyoA) displays a significant difference in the measured value in comparison to that which is expected, being 1830 Da smaller than predicted. This was observed for two independent preparations of the enzyme. This observation suggests that CyoA (subunit II) is post-translationally processed. Cytochrome bo_3 has been engineered with a histidine tag at the C-terminus of subunit II, and the presence of this tag is required to purify the enzyme using affinity chromatography (Rumbley et al., 1997). Hence, any proteolytic modifications of subunit II cannot remove the C-terminus, which contains the histidine tag, and must, therefore, involve the N-terminus, which has been shown previously to be blocked (Minghetti et al., 1992).

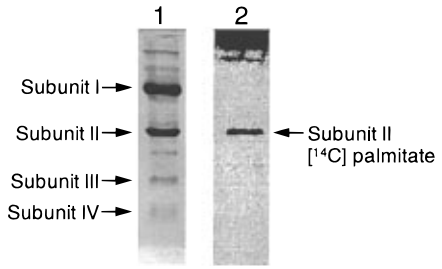

FIGURE 1: SDS−PAGE analysis of purified cytochrome bo_3 and demonstration of the incorporation of [^{14}C]palmitate into subunit II. Lane 1, Coomassie-stained SDS−PAGE of purified cytochrome bo_3 (40 μg of protein). Subunits I, II, III, and IV are indicated by arrows. Lane 2, autoradiography of cytochrome bo_3 prepared from cells grown in the presence of [^{14}C]palmitic acid during early log phase. The intense band at the top of the gel (lane 2) is due to the aggregates of peptides caused by high concentration of imidazole (which has been removed from the sample in lane 1).

Table 2: Comparison of Molecular Masses (in Da) Determined by Mass Spectrometry with the Calculated Values

subunit	calculated[a]	observed[b]	difference (observed − calculated)
I	74377	74306	−71
II	34915	33085	−1830
III	22626	22615	−11
IV	12030	11984	−46

[a] The molecular weights of subunit I, II, III, and IV are calculated from the deduced amino acid sequences of cyoB, cyoA, cyoC, and cyoD, respectively. [b] Values determined by mass spectroscopy.

Comparison of the deduced N-terminal sequence of subunit II reveals considerable similarity with signal sequences of known lipoproteins (Table 3). Common structural features include one or more positively charged amino acid residues near the N-terminus (the "N domain"), followed by a stretch of hydrophobic residues (H domain) and a cysteine residue with several highly conserved residues surrounding it (C domain) (Hayashi & Wu, 1990; Inouye et al., 1983, Yamaguchi et al., 1988). Based on this sequence similarity and the above experimental observations, it was speculated that subunit II of cytochrome bo_3 oxidase is synthesized as a precursor, which is modified and processed in the same way as lipoproteins.

The structure of the lipo-amino acid at the amino terminus of the lipoprotein has been elucidated, as illustrated in the case of the Braun's lipoprotein present in the *E. coli* outer membrane (Braun, 1975). A diacylglyceride containing two ester-linked fatty acids is covalently attached to the side chain of the cysteine through a thioether bond, and the NH_2 group of the cysteine forms an amide bond with an additional fatty acid. The ester-linked fatty acids are similar in composition to those present in bulk phospholipids, and 65% of the amide-linked fatty acids present in the lipoprotein is palmitic acid (Braun, 1975). In the case of subunit II of cytochrome bo_3, if the three putative fatty acids attached to the N-terminal cysteine are assumed to be palmitic acid, the calculated molecular mass would be 33028 Da, which is about 57 Da smaller than the observed value. It is therefore reasonable to suppose that subunit II of cytochrome bo_3 is modified in

Table 3: Comparison of the N-Terminal Signal Sequence of Subunit II of *E. coli* Cytochrome bo_3 with Those of Known Lipoproteins and Other Quinol Oxidases[a]

Lipoprotein	Signal Sequence		Reference
Braun's lipoprotein(LP) *E.coli*	MKATKLVLGAVILGSTLLA	CSSNA	(Nakamura & Inouye, 1979)
LP *S.Marcescens*	MNRTKLVLGAVILGSTLLAG	CSSNA	(Nakamura & Inouye, 1980)
LP *E.amylovora*	MNRTKLVLGAVILGSTLLAG	CSSNA	(Yamagata et al., 1981)
LP *M.morganii*	MGRSKIVLGAVVLASALLAG	CSSNA	(Huang et al., 1983)
LP *P.mirabilis*	MKAKIVLGAVILASGLLAG	CSSSN	(Ching & Inouye, 1986)
Tra t *protein E.coli*	MKMKKLMMVALVSSTLALSG	CGAMS	(Ogata et al., 1982)
Pullulanase *K.pneumoniae*	MLRYTCNALFLGSLILLSG	CDNSD	(Chapon & Raibaud, 1985)
Pullulanase *K.aerogenes*	MLRYTCHALFLSSLVILSG	CDNSS	(Katsuragi, 1987)
LP-28 *E.coli*	MKLTTHHLRTGAALLLAGILLAG	CDQSS	(Yu et al., 1986)
PenP *B.licheniformis*	MKLWFSTLKLKKAAAVLLFSCVALAG	CANNQ	(Neugebauer et al., 1981)
Cyt *R.viridis*	MKQLIVNSVATVALASLVAG	CFEPP	(Weyer et al., 1987)
Chitobiase *V.harveyi*	MLKHSLIAASVITTLAG	CSSLQ	(Soto-Gil & Zyskind, 1989)
Glucanase *P.solanacearum*	MRRCMPLVAASVAALMLAG	CGGGD	(Huang et al., 1989)
Oxidase *E.coli*	MRLRKYNKSLGWLSLFAGTVLLSG	CNSAL	(Chepuri et al., 1990)
Oxidase *B.subtilis*	MVIFLFRALKPLLVLALLTVVFVLGG	CSNAS	(Santana et al., 1992)
Oxidase *A.aceti*	MKNKLLARVARLGGLSSALLLAG	CELDV	(Fukaya et al., 1993)
Oxidase *P.denitrificans*	MTYIRKFARLPWLALLIPLAA	CKAEV	(Richter et al., 1994)

[a] The bottom four rows show the deduced N-terminal sequences of subunit II of the four currently available bacterial quinol oxidases.

Table 4: Properties of Mutants with an Altered Signal Sequence of Subunit II

mutant	oxidase activity (e^-/s)[a]	relative activity (%)	expression[b] (%)	N-terminal processing	K_M(ubiquinol-1) (μm)
WT	974	100	100	yes	49
R2L/R4L	988	110	89	yes	41
K5N/K8N	1039	116	72	yes	53
R4S/K5N/K8N	1075	120	69	yes	39
R2S/R4S/K5N/K8N	1030	115	66	yes	34
R2L/R4L/K5I/K8I	1070	119	54	no	37
C25A	545	56	66	no	56

[a] Results are expressed as electrons per second per CO-binding unit, using an extinction coefficient of 135 mM^{-1} cm^{-1}. [b] Determined by the relative CO-binding units in the membrane samples.

some manner similar to this, though definitive structural data are required to prove this.

Quantitation of the free sulfhydryls in pure cytochrome bo_3 was performed using Ellman's reagent (Ellman, 1959), and the results showed no reactive sulfhydryls in the native enyzme, but eight reactive cysteines after denaturation (not shown). The lack of reactivity of the sulfhydryls in the native enzyme is due to their being inaccessible and not due to the presence of disulfides. Reducing conditions were not used during the protein denaturation. The deduced sequence predicts a total of nine cysteine residues in subunits I, II, III, and IV, suggesting posttranslational modification or removal of a single cysteine residue in the enzyme.

If CyoA is processed as a lipoprotein, then fatty acids should be incorporated in the enzyme at position Cys25, which is the expected N-terminus of the mature subunit II by analogy with other lipoproteins. When [^{14}C]palmitic acid is added to the growth medium of *E. coli*, subunit II in the isolated cytochrome bo_3 is covalently labeled with the fatty acid. In this case, the histidine-tagged version of the enzyme was used to facilitate the isolation of the enzyme by affinity chromatography, and the eluted enzyme was analyzed by SDS−PAGE. Figure 1 shows the image obtained from scanning the electrophoresis gel with a PhosphoImager. A band is clearly observed with an apparent molecular mass of approximately 35 kDa, corresponding to subunit II with a histidine tag attached to its C-terminus. The broad and intense band at the top of the gel is due to the aggregates of cytochrome bo_3 oxidase formed under the conditions of the experiment.

Signal peptidase II recognizes only diglyceride-modified cysteine to process the signal sequence (Hayashi & Wu, 1990), and the requirement of a cysteine residue at the cleavage site for processing prolipoprotein has been demonstrated previously (Inouye et al., 1983). This was tested for CyoA by making the C25A mutant. The resulting oxidase is functional, with about half of the specific activity and half of the level of expression observed with the wild-type oxidase (Table 4). Western immunoblotting using antibodies directed against subunit II shows that subunit II appears to have a higher molecular mass compared to the wild type (Figure 2). This confirms that Cys25 is required for proper processing of CyoA and, furthermore, shows that the posttranslational processing is not essential for function. In addition, the K_m was determined for the substrate ubiquinol-1, and was found to be identical to that of the wild type. Hence, the processing is not involved in modulating the interaction with the quinol substrate.

Several other mutants were generated to examine the importance of the N-terminal signal sequence of CyoA. These are listed in Table 1, and the results are summarized in Table 4 and in Figure 2. The deletion of Arg2 to Ser9 (ΔR2S9) eliminates the incorporation of the oxidase in the membrane as judged by Western immunoblotting, enzyme

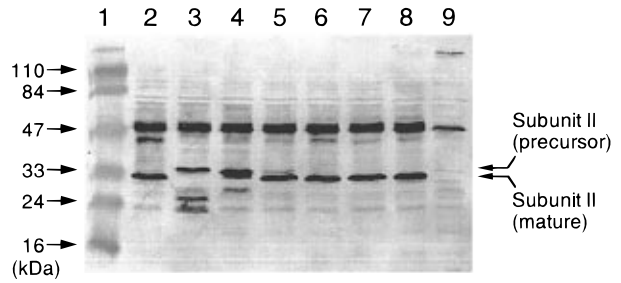

FIGURE 2: Western immunoblot analysis of membrane samples using strip-purified polyclonal antibodies directed against subunit I and subunit II. The mutations are identified in Table 1. Lane 1, prestained molecular mass standards; lane 2, wild-type enzyme; lane 3, C25A; lane 4, R2L/R4L/K5I/K8I; lane 5, R2S/R4S/K5N/K8N; lane 6, R4S/K5N/K8N; lane 7, K5N/K8N; lane 8, R2L/R4L; lane 9, ΔR2S9. All lanes, except lanes 1 and 9, were loaded with 10 μg of membrane protein. Lane 9 was loaded with 30 μg of membrane protein. The major bands that are observed are subunit I (apparent M_r 50 000) and subunit II variants (labeled). The band just under subunit I in most of the samples either may result from proteolysis of subunit I or may be a cross-reacting polypeptide in the membrane. Subunit II has the same mobility in the mutants in lanes 5−8 as does the wild-type oxidase (lane 2). The C25A mutation (lane 3) results in a distinctly larger size for subunit II (precursor), and significant proteolytic degradation of subunit II is apparent. To a lesser extent, the same is true for the mutant in lane 4 (see text).

activity, and spectroscopic criteria. In all other cases, the enzyme assembles in an active form and can support growth of the cells on nonfermentable growth substrates. When all four of the positively charged residues near the N-terminus (Arg2, Arg4, Lys5, and Lys8; see Table 1) are replaced by hydrophobic residues, the processing of subunit II is perturbed, according to the Western immunoblot (Figure 2), but the enzyme is fully active (Table 4). Spectroscopic analysis showed no differences between any of the mutants and the wild-type oxidase (not shown), so no perturbation of the heme environments is indicated.

DISCUSSION

In this work, it is clearly shown that subunit II (CyoA) of the cytochrome bo_3 oxidase from *E. coli* has an N-terminal signal sequence (von Heijne, 1990; Arkowitz & Bassilana, 1994) that is removed posttranslationally, as previously postulated (Chepuri et al., 1990; Minghetti et al., 1992). This is highly unusual for an intrinsic component of the cytoplasmic membrane. Furthermore, the data strongly indicate that the N-terminus of the mature polypeptide is Cys25 to which lipids have been covalently attached, typical of other lipoproteins in the bacterial envelope. This is consistent with the molecular mass of wild-type subunit II as determined by mass spectrometry, with the incorporation of palmitic acid into subunit II, and also with the increase of the apparent size of the subunit due to the C25A mutation.

Ichihara and his colleagues (Ichihara et al., 1981) have detected several lipoproteins in *E. coli*, in addition to the major lipoprotein in the outer membrane (Braun's lipoprotein). Two lipoproteins (NLP3 and NLP4) have been shown to be present in the cytoplasmic membrane of *E. coli*. NLP4 was later identified as the lpp-28 lipoprotein (Yu et al., 1986). It is most likely that subunit II of cytochrome bo_3 is the NLP3 lipoprotein previously reported by Ichihara (Ichihara et al., 1981). Unlike other lipoproteins localized in the inner membrane, subunit II does not have an aspartate residue following the modified terminal cysteine (Matsuyama et al., 1995; Seiffer et al., 1993). This may be related to the fact that CyoA is already anchored in the inner membrane by two transmembrane helices.

Cys25 is required for the cleavage of the signal peptide to form the mature subunit II, in agreement with the requirement of a cysteine residue at the cleavage site for processing prolipoproteins by signal peptidase II, as previously reported for Braun's lipoprotein (Inouye et al., 1983). The posttranslational modification of subunit II is not essential for either the assembly or the activity of the enzyme, however. The deletion of the signal peptide (residues 2 through 9) does eliminate the oxidase from the membrane, presumably by interfering with proper assembly. Interestingly, changing the four basic residues in the signal peptide to neutral residues does not necessarily result in the abolishment of processing. Two quadruple mutants (R2S/R4S/K5N/K8N and R2L/R4L/K5I/K8I; Tables 1 and 4), in which there are no charged residues in the N-domain of the signal peptide, are still able to assemble over 50% of the cytochrome bo_3 oxidase in the inner membrane relative to that of the wild-type control, although the N-terminal processing is absent in R2L/R4L/K5I/K8I. The role played by the positively charged amino acids in the N domain of signal peptides in the membrane translocation of exported proteins has been explored in detail in other systems (Puziss et al., 1989; Szczesna-Skorupa & Kemper, 1989; Vlasuk et al., 1983). The results obtained in these studies generally show that a net positive charge in the N domain is not absolutely required for the function of a signal peptide, but that substantial defects in the translocation can result when the net charge of the N domain becomes negative. Previous studies of the signal sequence of a virus glycoprotein have suggested that an upper limit of hydrophobicity may exist for eukaryotic signal sequences and exceeding this value could lead to an export defect (Tomilo et al., 1994). This might explain the apparently perturbed processing and proteolytic instability of the R2L/R4L/K5I/K8I mutant, since the signal sequence in this mutant is very hydrophobic.

Other ubiquinol oxidases also appear to undergo posttranslational processing of subunit II. This has been shown for both the *Paracoccus denitrificans* quinol oxidase (Richter et al., 1994) and *Acetobacter aceti* quinol oxidase (Fukaya et al., 1993). Sequence comparisons of other quinol oxidases show similar N-terminal extensions on subunit II as that in the *E. coli* oxidase (Table 3). There is no evidence, however, of covalent attachment of lipids in these other oxidases. Subunit II of the caa_3-type cytochrome *c* oxidase from *Bacillus firmus* also has been shown to have an N-terminal cysteine after processing, and it was in this report that the sequence similarity with lipoproteins was first noted (Quirk et al., 1993).

In summary, the data show that subunit II of the cytochrome bo_3 oxidase is a lipoprotein component of the cytoplasmic membrane. The posttranslational processing, however, is not necessary for either the assembly or function of the enzyme. It appears to play no role in the interaction with the lipophilic ubiquinol substrate. Neither is the covalently attached lipid necessary for anchoring the subunit to the membrane, since the polypeptide has two transmembrane helices (Chepuri et al., 1990; Iwata et al., 1995). Elucidating the functional and/or structural role of the modification will require further studies.

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