Involvement of a Putative [Fe-S]-cluster-binding Protein in the Biogenesis of Quinohemoprotein Amine Dehydrogenase*

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Quinohemoprotein amine dehydrogenase (QHNDH) of Paracoccus denitrificans contains a peptidyl quinone cofactor, cysteine tryptophylquinone, as well as intrapeptidyl thioether cross-links between Cys and Asp/Glu residues within the smallest \( \gamma \)-subunit of the \( \alpha \beta \gamma \) heterotrimERIC protein. A putative [Fe-S]-cluster-binding protein (ORF2 protein) encoded between the structural genes for the \( \alpha \) and \( \gamma \)-subunits of QHNDH in the \( n \)-butylamine-utilizing operon likely belongs to a Radical SAM (S-Ado-Met) superfamily that includes many proteins involved in vitamin biosynthesis and enzyme activation. In this study, the role of ORF2 protein in the biogenesis of QHNDH has been explored. Although the wild-type strain of Paracoccus denitrificans produced an active, mature enzyme upon induction with \( n \)-butylamine, a mutant strain in which the ORF2 gene had been mostly deleted, neither grew in the \( n \)-butylamine medium nor showed QHNDH activity. When the mutant strain was transformed with an expression plasmid for the ORF2 protein, \( n \)-butylamine-dependent bacterial growth and QHNDH activity were restored. Site-specific mutations in the putative [Fe-S]-cluster or SAM binding motifs in the ORF2 protein failed to support bacterial growth. The \( \alpha \) and \( \beta \)-subunits were both detected in the periplasm of the mutant strain, whereas the \( \gamma \)-subunit polypeptide was accumulated in the cytoplasm and stained negatively for redox-cycling quinone staining. Matrix-assisted laser desorption ionization time-of-flight mass spectrometric analysis revealed that the \( \gamma \)-subunit isolated from the mutant strain had not undergone posttranslational modification. These results unequivocally show that the putative [Fe-S]-cluster- and SAM-binding ORF2 protein is necessary for the posttranslational processing of \( \gamma \)-subunit, most likely participating in the formation of the intrapeptidyl thioether cross-links.

Quinohemoprotein amine dehydrogenase (QHNDH)\(^4\) is produced in the periplasmic space of certain Gram-negative bacteria, such as Paracoccus denitrificans and Pseudomonas putida, in response to primary amines, including \( n \)-butylamine and benzylamine, added to the culture medium as a sole carbon and energy source and catalyzes their oxidative deamination (1–3). QHNDH contains a posttranslationally derived quinone cofactor, cysteine tryptophylquinone (CTQ) (Fig. 1A), within the smallest \( \gamma \)-subunit of the \( \alpha \beta \gamma \) heterotrimERIC protein (4–6). The largest \( \alpha \)-subunit folds into four domains containing two \( \delta \)-type hemes in the first domain (5, 6). The \( \beta \)-subunit has a seven-bladed \( \beta \)-propeller structure often observed in quinoproteins. CTQ is the most recently identified, fourth quinone cofactor derived from an amino acid residue(s) constituting the polypeptide chain after the first identified topa quinone of copper amine oxidase (7), the second identified tryptophan tryptophylquinone (TTQ) (Fig. 1B) of methylamine dehydrogenase (MADH) (8), and the third identified lysine tyrosylquinone of lysyl oxidase (9). In addition to CTQ that is essential as a redox cofactor for the catalytic reaction (10, 11), the \( \gamma \)-subunit of QHNDH contains unique intrapeptidyl cross-links formed between the side chains of Cys and Glu or Asp residues (4–6) (Fig. 1C). Thus, sulfur atoms of the four Cys residues contained in the \( \gamma \)-subunit are all involved in the theioether cross-links with an indole ring carbon atom of Trp residue (in CTQ) and methylene carbon atoms of Glu or Asp residue, which are chemically very inert. It is reasonable to assume that the multiple cross-link structure contributes to the folding of the short \( \gamma \)-subunit polypeptide containing very few \( \alpha \)-helices but mostly coils (Fig. 1D). However, neither the functional role of the cross-links nor the mechanism of their posttranslational formation has so far been elucidated.

The genes coding for the three subunits of QHNDH appeared to constitute an operon in the genome of P. denitrificans which consists of at least 5 open reading frames (ORF1–5); the \( \alpha \), \( \beta \), and \( \gamma \)-subunits of QHNDH are encoded in ORF1, ORF4, and ORF3, respectively (5). The ORF5 protein is putatively an alkaline serine protease based on the homology search in a protein sequence database, but its role in the operon is unknown. The ORF2 protein encoded intervening between the \( \alpha \) and \( \gamma \)-subunit genes of QHNDH is also hypothetical but has been noted to belong to a Radical SAM (S-adenosylmethionine) superfamily that includes many proteins involved in vitamin biosynthesis and enzyme activation containing [Fe-S]-cluster and SAM binding motifs (12). The ORF2 protein is also encoded within the QHNDH genes of P. putida in the same gene array as in P. denitrificans (4). Enzymes belonging to the Radical SAM superfamily are thought to catalyze chemically difficult reactions, such as dithiobiotin synthesis from dethiobiotin (sulfur atom insertion) through the highly controlled free radical chemistry (13). Considering that the theioether modifications at methylene carbon atoms of carboxylic acid side chains in the \( \gamma \)-subunit of QHNDH would require an unusual chemical process to activate the very inert methylene carbon atoms, the putative [Fe-S]-cluster-binding ORF2 protein may participate in the posttranslational processing of the \( \gamma \)-subunit encoded in its immediate \( 3' \)-downstream.

In the studies herein reported we have focused on the role of ORF2
protein in the biogenesis of QHNDH, particularly in the posttranslational processing of the γ-subunit containing the CTQ cofactor and thioether cross-links. As a first step we have constructed a mutant strain of *P. denitrificans* lacking the most part of the ORF2 gene. The mutant strain did not grow in the medium containing an amine without showing the QHNDH activity, but its growth was rescued by transformation...
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with an expression plasmid for the ORF2 protein. Site-specific mutations in the putative [Fe-S]-cluster- or SAM binding motif of ORF2 failed to support the bacterial growth and to restore the QHNDH activity. Furthermore, the γ-subunit polypeptide accumulated in the cytoplasm of the mutant strain was found not to have undergone any post-translational modifications. Collectively, it is strongly suggested that the putative [Fe-S]-cluster-binding ORF2 protein plays an essential role in the posttranslational processing of the γ-subunit of QHNDH.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction endonucleases, DNA modifying enzymes, and endoprotease Asp-N were purchased from Takara Bio. All primers used in PCR were designed based on the nucleotide sequence reported previously for the QHNDH genes (5) (GenBank™ accession number AB063330) and purchased from GENSET OLIGO. A broad-host-range vector pBBR122 and pUC4K were provided from the National Institute of Genetics (Mishima, Japan). A suicide vector pGPRd1 (14) was kindly provided from Dr. R. J. van Spanning (Vrije Universiteit, The Netherlands). All other chemicals were of the highest grade commercially available.

**Bacterial Strains and Growth Conditions**—P. denitrificans IFO12442 was purchased from the Institute for Fermentation, Osaka (Osaka, Japan). P. denitrificans Pd1222 and Escherichia coli S17–1 (15) were also kindly provided from Dr. van Spanning and used as a host for gene disruption and a donor for transferring the suicide vector, respectively. E. coli DH5a and SCS110 were used for manipulating DNA and producing unmethylated plasmid DNA, respectively. E. coli strains were grown in the Luria broth (LB) medium at 37 °C. P. denitrificans strains were grown aerobically at 30 °C in the LB medium or in a mineral medium containing 15.14 g/liter Na₂HPO₄·12H₂O, 3.0 g/liter KH₂PO₄, 0.5 g/liter NaCl, 1.0 g/liter NH₄Cl, 0.5 g/liter yeast extract, 1 mM MgSO₄, 0.1 mM CaCl₂, 73 μM Na₂MoO₄, 1.6 μM CuSO₄, 20 μM Fe(III) citrate, and 0.5% glucose. Bacterial strains were cultured separately in 2 ml of the LB medium to a logarithmic phase, harvested by centrifugation, and resuspended in 200 μl of the medium. A mixture of the donor cell suspension (50 μl) and the recipient cell suspension (100 μl) was spread onto an LB agar plate. After incubation for 48 h at 30 °C, the cells were collected, and its appropriate dilutions were plated again on LB agar plates supplemented with rifampicin to kill the donor cells; P. denitrificans was rifampicin-resistant.

After further incubation for 48 h at 30 °C, clones having insertion mutations at the ORF2 and ORF3 gene loci were isolated using kanamycin and streptomycin (pGRPD1-derived) resistance as a double marker. To complete the process of chromosomal gene replacement, second recombinant was induced in the selected clones by growing on an LB agar plate containing only kanamycin. The resulting colonies were further examined for streptomycin sensitivity. The kanamycin-resistant and streptomycin-sensitive colonies for disruption of ORF2 gene were then subjected to colony PCR using primers P7 (5'-CCACCTGTTCAGATTCCGGCTGAGG-3') and P8 (5'-CTTCCCCAGCCCGGATCTGAACGAGTGG-3'), which were designed to amplify a 2.1-kb fragment including the kanamycin-resistant gene. Similarly, a 1.7-kb fragment including the kanamycin-resistant gene was amplified by colony PCR for ORF3 disrupted using primers P9 (5'-CAAGGCGAAAGCCCTTGGCAG-3') and P10 (5'-ATGCCAGGACATAGCGATT-3'). The amplified fragments were cloned into a pGEM-Easy vector and sequenced to confirm the insertion of the kanamycin-resistant gene cassette inside the ORF2 or ORF3 gene. Subsequently, the kanamycin resistance gene was deleted from the pKO-ORF2::Km' strain by conjugation using the second suicide vector, pKO-ΔORF2. After similar conjugation procedures, several clones were isolated using streptomycin resistance as the first index followed by using streptomycin-sensitive as the second index. The resultant colonies were subjected to colony PCR to confirm the deletion of the kanamycin-resistant gene. A positive clone with only a 0.9-kb fragment amplified due to the gene deletion was designated PdKO4. The amplified fragment was also cloned into a pGEM-Easy vector and sequenced to confirm the deletion. The Pd1222 strain carrying ORF3: Km' was designated PdKO6 and used without removal of the kanamycin resistance gene.
Construction of Expression Plasmids—An EcoRI fragment containing the region from the 5′-upstream non-coding sequence of the ORF1 gene to the 3′-terminus of ORF4 gene was excised from pKO3 and ligated into pBRR122 to yield pKO11, a plasmid for expression of the entire QHNDH proteins (ORF1–4) in P. denitrificans. To construct a expression plasmid for only the ORF2 protein, the ORF2 gene was amplified by PCR using the above-constructed pGem-T containing the entire QHNDH gene as a template with primers P11 (5′-ATCCATATGCAGGGTGGCTACGCTCATCCTC-3′) and P12 (5′-CCGCTTTGGAAGGGAACCTCCCT-TGC-3′) in which NdeI and BamHI sites (underlined) were introduced, respectively. The ORF2 gene was amplified and inserted into pKO11 at the NdeI/BamHI sites (replacing the ORF1–4 genes) to yield pKO12-ORF2. Furthermore, to facilitate detection of the plasmid-derived ORF2 protein, a hexahistidine (His6) epitope tag with a linker sequence (GGGGSDHHHHHH) was attached to the C terminus of the ORF2 protein as follows. The ORF2 gene in pKO12-ORF2 was again amplified by PCR with primers P11 and P13 (5′-GATCATCCATATGCAGGGTGGCTACGCTCATCCTC-3′ and P15 (5′-GATCTTGGATGTCGATGTGGATGTCGATGAT-3′) containing a BamHI site (underlined), and the fragment amplified was cloned into pGem-T Easy. A 5′-protruding, double-stranded DNA fragment encoding the linker sequence produced by annealing of two phosphorylated oligonucleotides, P14 (5′-GATCATCCATATGCAGGGTGGCTACGCTCATCCTC-3′) and P15 (5′-GATCTTGGATGTCGATGTGGATGTCGATGAT-3′) was inserted into the BamHI site of the plasmid obtained above. Then the NdeI/BamHI fragment was excised and subcloned into pKO11 as above to yield pKO15-ORF2His for expression of the His6-tagged ORF2 protein. This plasmid was also used as the template for site-specific mutagenesis described below.

Two kinds of expression plasmids for the ORF3 gene encoding γ-subunit were constructed as follows. A 0.33-kb fragment covering from the Met codon at the newly identified −28 position (see “Results”) to the Ser-82 codon (long version) and a 0.25-kb fragment covering from the original Met1 codon to the Ser-82 codon (short version) were separately amplified by PCR using the plasmid containing a 4.4-kb fragment of the QHNDH gene as a template with sense strand primers P16 (5′-GGAGGAGGTCTCCATATGAAAATCATTGCAC-3′ (for the long version)) and P17 (5′-GGAGGAGGTCTCCATATGAATTGCCTGGTGG-3′ (for the short version)), containing an NdeI site (underlined), and a common antisense strand primer, P18 (5′-GATCTTGGAGAGGAGGATGTCGATGATGTCGATGATGTTCC-3′), containing a BamHI site (underlined). The amplified DNA fragments were digested with NdeI and BamHI, and the resultant 0.33- and 0.25-kb fragments were inserted into pKO12 at the NdeI/BamHI sites (replacing the ORF2 gene). Finally, the EcoRI fragments containing the expression cassettes for the long and short versions of γ-subunit were excised and ligated into an EcoRI site of pRK415-1, carrying a tetracycline-resistant gene for plasmid maintenance (16), to yield pKO15-ORF2His-γ (replacing the wild-type ORF2 gene). The mutant plasmids thus obtained (pKO15-C131S, pKO15-G176A, and pKO15-C427S) were used to transform the PdKO4 strain for evaluating the effects of mutagenesis on the cell growth utilizing n-butylamine as a sole source of carbon and energy. Expression of the wild-type and mutant His6-tagged ORF2 proteins in P. denitrificans was examined by Western blotting with an antibody against the His6 tag as described later.

Enzyme Purification—Plasmid pDS208 for expression of the recombinant QHNDH in P. denitrificans under the control of the cytochrome c promoter (cyccA) was constructed by cloning the entire QHNDH gene (ORF1–4), in which a His6 tag sequence was added at the C terminus of the β-subunit for easier purification of the enzyme by an affinity method, into plasmid pRK415-1 (16). P. denitrificans PdKO4 cells transformed with pDS208 by electroporation were grown aerobically at 30 °C in the LB medium supplemented with 1 µg/mL tetracycline. A 5-mL portion of the cultured cell suspension was inoculated into the mineral medium (generally 500 mL) supplemented with 0.5% n-butylamine and 1 µg/mL tetracycline. Under these conditions both of the recombinant QHNDH derived from the plasmid and the native one derived from the host cell genome were produced simultaneously; about 40% was estimated to be the recombinant His6-tagged enzyme as judged from the amount of QHNDH activity adsorbed onto a nickel nitrilotriacetic acid column. After 24 h the cells were harvested by centrifugation, and the periplasmic fraction was isolated as described below. A nickel nitrilotriacetic acid Superflow resin (Qiagen) preequilibrated with 20 mM potassium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole (wash buffer) was mixed with the periplasmic fraction, and the mixture was stirred gently for 30 min at 4 °C and then poured into a chromatography column. After the column was washed thoroughly with the wash buffer, the His6-tagged enzyme was eluted with 20 mM potassium phosphate buffer containing 300 mM NaCl and 70 mM imidazole, pH 8.0. The active fractions were combined and dialyzed at 4 °C against 10 mM potassium phosphate buffer, pH 7.5, containing 50 mM NaCl. After the enzyme solution was centrifuged at 50,000 × g for 60 min, the clear supernatant was applied to a Mono Q HR10/10 column (Amersham Biosciences) equipped on an Amersham Biosciences fast protein liquid chromatography system preequilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 50 mM NaCl (buffer A). After the column was thoroughly washed with buffer A, the enzyme was eluted with a 160-mL linear gradient of 0.05–0.5 M NaCl in buffer A. The purified enzyme was concentrated, dialyzed against buffer A, and stored at 4 °C until use.

Preparation of Cell-free Extracts—P. denitrificans cells were harvested from 10 mL of the culture medium and resuspended in 20 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA/2Na and 0.25 mg/mL lysozyme. The cells were disrupted by sonication (Tomy, UD-201), and the supernatant obtained by centrifugation at 20,000 × g for 20 min was pooled as the cell-free extract. The cells were also fractionated into the periplasmic and cytoplasmic fractions essentially according to the method reported by Alefounder and Ferguson (17). Briefly, the cells were suspended in 20 mM potassium phosphate buffer, pH 7.5, containing 0.5 M sucrose and 0.5 mM EDTA/2Na. After the addition of 5 mg of lysozyme (wt) of the cells and 1 mM phenylmethylsulfonyl fluoride (protease inhibitor), the cell suspension was incubated at 30 °C for 30 min with gentle swirling. The periplasmic fraction was obtained in the supernatant after centrifugation at 20,000 × g for 20 min. The precipitated spheroplasts were resuspended at 0.1 g/mL of 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA/2Na. After the spheroplasts were disrupted by sonication, the
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supernatant obtained by centrifugation at 20,000 × g for 20 min was used as the cytoplasmic fraction.

Enzyme and Protein Assays—The QHNDH activity was measured in 20 mM potassium phosphate buffer, pH 7.5, containing 50 mM 2,6-dichlorophenolindophenol, 1 mM phenazine methosulfate, and 10 mM n-butylamine-HCl as substrate, monitoring the decrease in absorbance at 600 nm due to the reduction of 2,6-dichlorophenolindophenol (1). One unit of the enzyme activity was defined as the amount that reduces 1 μmol of 2,6-dichlorophenolindophenol per min. Protein concentration was determined by the method of Bradford (18) using bovine serum albumin as the standard.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis was performed under the standard conditions (19) using 12 and 15% gels for separation of α/β-subs units and γ-subunit, respectively. Before electrophoresis protein samples were denatured thoroughly as reported previously (5). For Western blotting, proteins were electrotransferred to Immobilon-P and Immobilon-PSQ (Millipore) polyvinylidene difluoride membranes for detection of α/β-subs units and the γ-subunit, respectively, at 15 V for 30 min using a standard blotting buffer (20) except for using 5% methanol. The α- and β-subs units of QHNDH were detected by incubation with a 1:5000 dilution of an anti-QHNDH polyclonal antiserum that had been raised in BALB/c mice in a blocking buffer consisting of 5% (w/v) skim milk and 0.05% (v/v) Tween 20 in phosphate-buffered saline followed by incubation with a 1:10,000 dilution of the horseradish peroxidase-conjugated secondary anti-mouse sheep antibody (Amersham Biosciences). The γ-subunit was detected by incubation with a 1:500 dilution of an anti-γ-polypeptide polyclonal antiserum that had been raised in white rabbits using a synthetic oligopeptide corresponding to residues 71–81 of the γ-subunit followed by incubation with a 1:10,000 dilution of the horseradish peroxidase-conjugated secondary anti-rabbit donkey antibody (Amersham Biosciences). The ORF2 protein was also detected by incubation with a 1:5,000 dilution of an anti-ORF2 protein polyclonal antiserum that had been raised in white rabbits using the ORF2 protein expressed in the insoluble fraction of E. coli BL21 (DE3) cells followed by incubation with a 1:10,000 dilution of the horseradish peroxidase-conjugated secondary anti-rabbit donkey antibody. To eliminate antibodies against contaminating proteins derived from E. coli cells, the anti-ORF2 protein antiserum was pretreated overnight at 4 °C with an equal volume of 10% (w/v) aceton-dried powder of E. coli BL21 (DE3) cells suspended in phosphate-buffered saline containing 0.05% (v/v) Tween 20. The His4-tagged ORF2 protein was detected by incubation with a 1:5,000 dilution of an anti-His4 mouse polyclonal antibody (Qiagen) followed by incubation with a 1:10,000 dilution of the horseradish peroxidase-conjugated secondary anti-mouse sheep antibody (Amersham Biosciences). Protein bands were detected with ECL Plus Western blotting detection reagents (Amersham Biosciences) according to the manufacturer’s instructions and visualized with a ChemiDoc XRS system (Bio-Rad). The band intensity was quantified using the Quantity One Version 4.5.0 software (Bio-Rad).

Quinone Staining—The redox-cycling quinone staining was carried out by the method of Paz et al. (21) with a minor modification. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-PSQ). Quinone group was detected by staining the polyvinylidene difluoride membrane with 2 mM potassium glycinate, pH 10.0, containing 0.24 mM nitro blue tetrazolium. The membrane was immersed in the solution for 45 min in the dark.

Isolation of γ-Subunit Polypeptide—P. denitrificans PdKO4 was cultured in the medium containing n-butylamine and choline, and the spheroplasts prepared as described above were washed twice with phosphate-buffered saline and resuspended at a final concentration of 0.1 g/ml in 20 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM dithiothreitol and 10 mM EDTA-2Na. The spheroplasts were disrupted by sonication, and the supernatant was obtained by centrifugation at 50,000 × g for 60 min. Ice-cold acetone was added to the supernatant to a final concentration of 65% (v/v), and the mixture was kept at −80 °C overnight. The solution was thawed and centrifuged at 50,000 × g for 60 min. The supernatant was concentrated by evaporation, and trichloroacetic acid was added to a final concentration of 10% (v/v). After centrifugation at 50,000 × g for 60 min, the precipitates were washed twice with ice-cold acetone. Residual acetone was dried away by blowing nitrogen gas, and the precipitates were then dissolved in 50% acetonitrile containing 0.2 M ammonium bicarbonate, 5 mM EDTA-2Na, and 50 mM Tris(2-carboxyethyl)phosphine-HCl, pH 7.0. After incubation at 55 °C for 30 min, 4-phenylpyridine was added to a final concentration of 0.1 M, and the solution was incubated at room temperature for 4 h. The S-pyridylethylated sample was centrifuged at 20,000 × g for 20 min, and the clear supernatant was dialyzed at 4 °C overnight against 50 mM ammonium bicarbonate in 20% acetonitrile. After the addition of formic acid to the dialyzed sample to a final concentration of 1.5% (v/v), insoluble aggregates were removed by centrifugation. The crude sample of γ-subunit polypeptide was purified on an HPLC system (Tosoh) using a C4 reverse phase column (Vydac, 4.6 × 150 mm) with a 30-min linear gradient of 16–40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. The fractions containing the γ-subunit polypeptide were detected by Western blotting as described above, and the positive fractions were concentrated with a centrifugal concentrator (Tomy, CC-180). The concentrate was further purified on an HPLC system using a C18 reverse phase column (Tosoh, TSK-gel Octadecyl-4PW, 4.6 × 150 mm) with a 50-min linear gradient of 25–36% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The fractions containing the γ-subunit polypeptide were collected and concentrated. The second HPLC separation was repeated again, and the resultant sample was used for mass spectrometric analysis. The γ-subunit polypeptide was also isolated from the purified recombinant QHNDH. The purified enzyme (10 μg) was dissolved in 50 μl of Tris-HCl buffer, pH 8.0, containing 7 μM guanidine-HCl and 10 mM EDTA-2Na. After incubation for 10 min at 95 °C, 850 μl of distilled water and 100 μl of trichloroacetic acid were added to the solution and kept at −80 °C for 1 h. The γ-subunit was precipitated by centrifugation and then washed twice with acetone. The precipitate was dissolved in 0.05% trifluoroacetic acid in 50% acetonitrile, and the resultant sample was used for mass spectrometric analysis.

Matrix-assisted Laser Desorption/Ionization—Time of Flight (MALDI-TOF) Mass Spectrometric Analysis—MALDI-TOF mass spectra were obtained on an Autoflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with a 337-nm nitrogen laser lamp. The samples were prepared by the drying droplet method, drying 1 μl each of sample and matrix solutions consisting of sinapic acid on a stainless steel target plate. Measurements were performed in the positive-ion linear mode with delayed extraction. For sequence analysis of the inner region of the γ-subunit, the purified γ-subunit polypeptide was dissolved in 50 μl of 50 mM ammonium bicarbonate in 50% acetonitrile and digested with 0.06 μg of endoproteinase Asp-N. After incubation at 37 °C for 16 h, the mixture was concentrated to about 10 μl and analyzed by MALDI-TOF mass analysis. The sample was prepared by the drying droplet method with α-cyano-4-hydroxycinnamic acid as the matrix. All MALDI spectra were initially calibrated with InvitomassTM low molecular weight mass calibrant kit (Invitrogen). Protein and pep-
expression levels of the ORF2 protein are induced by the addition of an alternative carbon and energy source, the bacterium grew efficiently by inducibly forming QHNDH (Fig. 2A), which catalyzes the 2e− oxidation of n-butylamine, as reported previously for the P. denitrificans IFO12442 strain (1). However, the PdKO4 mutant strain derived from Pd1222, whose ORF2 gene had been mostly deleted by homologous recombination, could neither produce the QHNDH activity nor grow well in the n-butylamine medium (Fig. 2A). Although the cell growth of PdKO4 strain was recovered significantly by the addition of an alternative carbon and energy source, choline (22–24), the QHNDH activity was scarcely induced (Fig. 2B). Western blot analysis of the cell-free extracts revealed that the expression levels of the α-, β-, and γ-subunit proteins of QHNDH were depressed considerably in the PdKO4 strain (to 2, 19, and 34% of those of the Pd1222 strain, respectively, based on the densitometric analysis) (Fig. 2, C and D). We found that the expression levels of β- and γ-subunit proteins increased significantly in the PdKO4 cells grown in the medium supplemented with choline, even without showing the QHNDH activity (Fig. 2, C and D). The reason for the low expression of the α-subunit protein in the choline-grown cells of PdKO4 (Fig. 2C) is unclear but may be due to its faster degradation than those of the other subunit proteins. The presence and absence of the ORF2 protein itself were confirmed in the cell-free extracts of the wild-type Pd1222 strain and the mutant PdKO4 strain, respectively, by Western blotting (Fig. 3A). It is also noted that the ORF2 protein was inducibly formed when the Pd1222 cells were grown in the n-butylamine medium but not in the non-inducing medium using glucose as an energy and carbon source (Fig. 3B), indicating that the expression of ORF2 protein is controlled under the same promoter as the QHNDH expression. These results clearly show that the ORF2 protein is not hypothetically but actually expressed as a protein to be involved in the biogenesis of active QHNDH that facilitates the bacterial cell growth in the n-butylamine medium. Disruption of the ORF2 gene leads to the prevention of the cell growth and the active QHNDH formation. Even if the growth of the PdKO4 cells is recovered by other nutrients, the subunit proteins of inactive QHNDH are merely accumulated within the cells. Thus, it is strongly suggested that the ORF2 protein participates in the posttranslational modification (activation) of QHNDH.

ORF2 gene is expected to function for expression of the plasmid-encoded QHNDH. Using a broad-host-range vector pBBR122, we have constructed plasmids for expression of the whole QHNDH proteins (ORF1–4) (pKO11) and the ORF2 protein with or without a His6 tag (pKO15-ORF2His and pKO12-ORF2, respectively) in P. denitrificans. An ~800-bp 5′-upstream non-coding region of the ORF1 gene has also been included in the expression plasmids, so that the original n-butylamine-inducible promoter sequence is sufficiently contained and expected to function for expression of the plasmid-encoded QHNDH and ORF2 genes as well. When the cells of PdKO4 strain lacking the ORF2 gene were transformed with the expression plasmid for QHNDH (pKO11) or ORF2 (pKO12-ORF2), both the cell growth and the QHNDH activity were significantly recovered in the medium contain-
Binding Motifs of ORF2

—It has been noted previously that the ORF2 gene-lacking PdKO4 cells were grown in the mineral medium supplemented with n-butylamine. A, cell growth measured by absorbance at 600 nm (○, □, △) and QHNDH activities in the cell-free extracts (○, □, △) were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □, △), pKO11 (○, □, △), or pKO12-ORF2 (○, □, △). B, cell growth measured by absorbance at 600 nm were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □), pKO12-ORF2 (○, □), pKO15-C131S (○, □), pKO15-C427S (○, □), and pKO15-G176A (○, □). Inset, the expressed ORF2 proteins in the extracts of cells grown for 12.5 h were analyzed by Western blotting using an anti-His4 antiserum.

FIGURE 4. Recovery of cell growth and QHNDH activity in PdKO4 mutant strain by the plasmid-encoded ORF2 gene. The ORF2 gene-lacking PdKO4 cells were grown in the mineral medium supplemented with n-butylamine. A, cell growth measured by absorbance at 600 nm (○, □, △) and QHNDH activities in the cell-free extracts (○, □, △) were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □, △), pKO11 (○, □, △), or pKO12-ORF2 (○, □, △). B, cell growth measured by absorbance at 600 nm were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □), pKO12-ORF2 (○, □), pKO15-C131S (○, □), pKO15-C427S (○, □), and pKO15-G176A (○, □). Inset, the expressed ORF2 proteins in the extracts of cells grown for 12.5 h were analyzed by Western blotting using an anti-His4 antiserum.

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—It has been noted previously that the ORF2 gene-lacking PdKO4 cells were grown in the mineral medium supplemented with n-butylamine. A, cell growth measured by absorbance at 600 nm (○, □, △) and QHNDH activities in the cell-free extracts (○, □, △) were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □, △), pKO11 (○, □, △), or pKO12-ORF2 (○, □, △). B, cell growth measured by absorbance at 600 nm were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □), pKO12-ORF2 (○, □), pKO15-C131S (○, □), pKO15-C427S (○, □), and pKO15-G176A (○, □). Inset, the expressed ORF2 proteins in the extracts of cells grown for 12.5 h were analyzed by Western blotting using an anti-His4 antiserum.

FIGURE 4. Recovery of cell growth and QHNDH activity in PdKO4 mutant strain by the plasmid-encoded ORF2 gene. The ORF2 gene-lacking PdKO4 cells were grown in the mineral medium supplemented with n-butylamine. A, cell growth measured by absorbance at 600 nm (○, □, △) and QHNDH activities in the cell-free extracts (○, □, △) were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □, △), pKO11 (○, □, △), or pKO12-ORF2 (○, □, △). B, cell growth measured by absorbance at 600 nm were plotted against cultivation time (h) for the PdKO4 cells carrying no plasmid (○, □), pKO12-ORF2 (○, □), pKO15-C131S (○, □), pKO15-C427S (○, □), and pKO15-G176A (○, □). Inset, the expressed ORF2 proteins in the extracts of cells grown for 12.5 h were analyzed by Western blotting using an anti-His4 antiserum.

Site-specific Mutagenesis in the Putative [Fe-S]-cluster and SAM Binding Motifs of ORF2—It has been noted previously that the ORF2 protein likely belongs to the Radical SAM superfamily (4, 5). Multiple sequence alignment with other Radical SAM proteins (12) revealed the presence of the motifs in the ORF2 protein; a Cys-rich region (127CNLACTYC134) exactly matching with a characteristic C presence of the following motifs in the ORF2 protein; a Cys-rich region C427S (pKO12-ORF2 (○, □, △), pKO15-C131S (○, □, △), pKO15-C427S (○, □, △), and pKO15-G176A (○, □, △)). These results confirm the essentiality of the ORF2 protein for the active QHNDH biogenesis and the n-butylamine-dependent bacterial growth.

Site-specific Mutagenesis in the Putative [Fe-S]-cluster and SAM Binding Motifs of ORF2—It has been noted previously that the ORF2 protein likely belongs to the Radical SAM superfamily (4, 5). Multiple sequence alignment with other Radical SAM proteins (12) revealed the presence of the motifs in the ORF2 protein; a Cys-rich region (127CNLACTYC134) exactly matching with a characteristic CXX-CXXC consensus sequence that is predicted to serve as an [Fe-S]-cluster binding site, a C-terminal Cys-rich region (428CKTCRIRSCAGGC457) also conserved in some Radical SAM proteins and assumed to be the binding motif for an additional [Fe-S] cluster or a metal ion(s), and a GGE motif (179GGE178) involved in binding of SAM. Site-specific mutagenesis of invariant amino acid residues in these putative [Fe-S]-cluster and SAM binding motifs of the ORF2 protein was employed for examining the ORF2 protein to function as a Radical SAM protein. Thus, two Cys residues, Cys-131 and Cys-427, in the Cys-rich regions and Gly-176 in the GGE motif were mutated into Ser and Ala residues, respectively, and the expression vectors (pKO15-C131S, pKO15-G176A, and pKO15-C427S) were constructed for the His6-tagged ORF2 protein having a C131S, C427S, or G176A mutation. Similar to the expression plasmid for the ORF2 protein without a His6 tag, the expression plasmid for the His6-tagged ORF2 protein without mutation recovered the growth of the transformed PdKO4 cells in the n-butylamine medium (Fig. 4B), indicating that the addition of the His6 tag with a linker to the C terminus of ORF2 protein has virtually no effect on the function of ORF2 protein. In contrast, the ORF2 proteins with mutations in the putative [Fe-S]-cluster and SAM binding motifs (C131S, C427S, and G176A) did not assist the growth of the ORF2-disruptant strain PdKO4 (Fig. 4B). Amounts of the expressed proteins of the ORF2 mutants in the cell extracts were comparable with that of ORF2 protein without mutation (wild type) (Fig. 4B, inset). These results strongly suggest that the ORF2 protein assists the n-butylamine-dependent growth of PdKO4 cells by functioning as a Radical SAM protein.

Detection of γ-Subunit in PdKO4 Cells and Its Isolation—The mature, active QHNDH was shown to be present in the periplasmic space of Gram-negative bacteria (1, 2). Consistent with this intracellular localization of QHNDH, nucleotide and amino acid sequence analyses revealed that both of the α- and β-subunits have N-terminally flanking signal sequences, which would direct translocation of the polypeptides into the periplasm (4, 5). Curiously, however, the γ-subunit had apparently no signal sequence; the amino acid sequence after the translation initiator Met predicted from the nucleotide sequence was identical with that determined by direct chemical sequencing (4, 5). The γ-subunit was, therefore, assumed to be translocated into the periplasm by tightly associating with the α-subunit (5). Indeed, Western blot analysis of the periplasmic and cytoplasmic fractions showed that most (93–95%) of the α-, β-, and γ-subunit polypeptides were present in the periplasm of the wild-type Pd1222 strain (Fig. 5, A and B). In the ORF2 gene-deleted PdKO4 strain producing inactive QHNDH by growing in the choline-supplemented medium (see Fig. 2), the α- and β-subunit polypeptides were also mostly detected in the periplasm, as in the wild-type strain (Fig. 5A). In contrast, the γ-subunit polypeptide produced by the PdKO4 cells was found to be accumulated in the cytoplasm (Fig. 5B). Therefore, the α- and β-subunit polypeptides were translocated into the periplasm irrespective of the γ-subunit polypeptide. In addition, the γ-subunit of the periplasmic QHNDH of the wild-type strain was stained positively by the redox-cycling method for detection of quinone groups, whereas that accumulated in the cytoplasm of PdKO4 was completely negative (Fig. 5C), indicating the absence of the redox-active quinone group. When the PdKO4 cells were rescued with the ORF2 gene, the γ-subunit disappeared from the cytoplasm but was detected in the periplasm (Fig. 5B) and became positive for quinone-staining (Fig. 5C). This result strongly suggests that the γ-subunit polypeptide accumulated in the cytoplasm of PdKO4 cells is the precursor of the γ-subunit constituting the active, periplasmic QHNDH. As for the ORF2 protein, it also has no sequence potentially functioning as a signal peptide (5) and has been shown here experimentally to reside within the cytoplasm of the wild-type Pd1222 strain (Fig. 3C).

To further analyze the extent of posttranslational modification of the γ-subunit polypeptide accumulated in the cytoplasm of the PdKO4 mutant strain (Fig. 5B), the γ-subunit was isolated from the cells cultured in the medium containing n-butylamine and choline by extraction with acetone and precipitation with trichloroacetic acid followed by HPLC using reverse-phase columns as described under “Experimental Procedures.” However, an initial attempt to isolate the γ-subunit by applying the acetone extract directly to a C4 reverse-phase column of
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FIGURE 5. Subcellular localization of QHNDH subunits. Wild-type Pd1222, mutant PdKO4 strains with or without the expression plasmid for ORF2 protein (pKO12-ORF2), and mutant PdKO6 strain with or without the expression plasmids for the long and short versions of γ-subunit (pKO30-ORF3-long and pKO30-ORF3-short, respectively) were grown for 24 h in the medium supplemented with n-butylamine and choline. Periplasm (Peri.) and cytoplasmic (Cyto.) fractions were prepared as described under “Experimental Procedures,” and proteins in each fraction were subjected to Western blotting using the antiserum raised against the whole enzyme for detection of the α- and β-subunits (A) or that raised against a synthetic oligopeptide for detection of the γ-subunit (B). The γ-subunit was also stained for redox-active quinone groups as described under “Experimental Procedures.” C, each lane was applied with about 5 μg (A) and 50 μg of total proteins (B and C).

HPLC was unsuccessful due to broadened elution of the γ-subunit polypeptide, detectable by Western blotting, in several unspecified fractions (data not shown). Assuming that the γ-subunit produced in the PdKO4 strain had not undergone posttranslational modification and, hence, might contain maximally 4 Cys residues with a free SH group (see Fig. 1C), which could produce complicated structures by forming intramolecular thiol-disulfide bridges, the acetone-extracted γ-subunit was treated with 4-vinylpyridine before HPLC to block the free SH groups. Consequently, the S-pyridylethylated γ-subunit was eluted sharply from the C4 reverse-phase column in a single fraction (data not shown), and finally could be purified to homogeneity by repeated HPLC with a C18 reverse-phase column as shown in Fig. 6A. On the other hand, the fully processed γ-subunit in the mature, active QHNDH could be isolated from the purified enzyme simply by precipitation with 10% trichloroacetic acid without HPLC as described under “Experimental Procedures.”

MALDI-TOF Mass Spectrometric Analysis of the γ-Subunit—MALDI-TOF mass analysis has shown that the mass of the fully processed γ-subunit is 8857.42 ± 5.95 Da (average mass), which is comparable with that estimated from the chemical and crystal structures including all post-translational modifications except for removal of the N-terminal Met residue (calculated mass, 8857.57 Da) (Table 1). In marked contrast, the S-pyridylethylated γ-subunit was eluted sharply from the C4 reverse-phase column in a single fraction (data not shown), and finally could be purified to homogeneity by repeated HPLC with a C18 reverse-phase column as shown in Fig. 6A. On the other hand, the fully processed γ-subunit in the mature, active QHNDH could be isolated from the purified enzyme simply by precipitation with 10% trichloroacetic acid without HPLC as described under “Experimental Procedures.”

MALDI-TOF mass fragment analysis provided the following information. 1) The endoprotease Asp-N-digested sample contained five peptide fragments assignable to the inner partial sequences of the γ-subunit: Asp-12—Ala-32 (peak 6), Asp-18—Ala-32 (peaks 3 and 4), Asp-33—Ala-38 (peak 1), Asp-39—Ala-48 (peak 2), and Asp-67—Ser-82 (peak 5). 2) All of the four Cys residues (Cys-7, Cys-27, Cys-41, and Cys-37) involved in the thioether linkage with the methylene carbon atoms of Asp or Glu residues (Glu-16, Asp-33, and Asp-49) and the indole carbon atom of Trp-43 in the mature γ-subunit had been S-pyridylethylated, indicating that their SH groups were all free, i.e. without forming thioether cross-links. 3) Conversely, the three acidic residues (Glu-16, Asp-33, and Asp-49) had not undergone modification at their methylene carbon atoms (although Asp-49 was not included in the identified peptide fragments, it was suggested that it had an unmodified side chain as judged from the Asp-N cleavage). 4) Similarly, the indole ring of Trp-43 was unmodified, i.e. neither cross-linked to Cys-37 nor oxidized to tryptophylquinone. 5) Met-30 was partially oxidized to methionine sulfoxide (in peak 4), probably due to oxidation during the isolation procedure of the γ-subunit. Collectively, these data indicate that the γ-subunit polypeptide accumulated in the cytoplasm of the ORF2 gene-lacking PdKO4 mutant strain had suffered none of the posttranslational modifications (CTQ and Cys-Asp/Glu thioether cross-links) found in the γ-subunit of the active, mature QHNDH.

More careful inspection of the mass fragment analysis revealed the presence of four peptide fragments with very small peak intensities at m/z 4250—4300 Da (peaks 7–10, Table 1) (Fig. 7A, inset). Initially, these peaks did not correspond to any part of the γ-subunit sequence and, therefore, were thought to be derived from a contaminating protein(s). However, when the assignment was extended to the entire QHNDH gene, the peak at m/z 4255.85 ± 0.64 Da was found to exactly match with an S-pyridylethylated 28-residue amino acid sequence deduced from the nucleotide sequence of the 5′-upstream non-coding region of the ORF3 gene plus 11 residues in the coding sequence of the γ-subunit. The following three peaks at m/z 4271.21 ± 0.04, 4286.95 ± 0.71, and 4300.97 ± 0.87
Da ([M+H]+, monoisotopic mass) were also assigned to the same peptide (residues from −28 to 11) but containing 1 or 2 methionine sulfoxides and 1 methionine sulfoxone (Table 1). Thus, all these four fragments were produced by the Asp-N cleavage at Asp-12 of the polypeptide but were N-terminally longer by 28 residues than the reported sequence of the γ-subunit (Fig. 7B). If the Met residue at the −28 position is taken as the new translation initiator, the mass of the γ-subunit polypeptide with the pre-sequence is calculated to be 12427.92 Da, including 4 pyridylethyl groups and 2 methionine sulfoxides. This value is very close to the experimental mass (m/z 12,423.36 ± 4.35 Da) determined for the γ-subunit polypeptide isolated from the cytoplasm of the PdKO4 cells (Table 1). Although the initial assignment of Met1 as the translation initiator was made on the basis of the chemical sequence and crystal structure analyses and also the absence of a typical signal sequence (4, 5), the translation initiator of the γ-subunit should be corrected to the Met residue at the −28 position.

**Disruption of γ-Subunit Gene and Rescue**—To establish the essentiality of the above identified 28-residue N-terminal extension of γ-subunit, we disrupted the chromosomal ORF3 gene by inserting the Km' gene cassette and examined the rescuing effects of the long and short
versions of the γ-subunit gene carried on a plasmid, coding for the regions from the newly identified Met at position 28 to the C terminus Ser-82 and from the original Met1 to Ser-82, respectively. The PdKO6 strain with the γ-subunit gene disrupted could not grow well in the n-butylamine medium. Although the mutant strain grew in the medium supplemented with both n-butylamine and choline, it showed only a minuscule QHNDH activity in the cell-free extract (0.003 units/mg, about 0.4% of the activity of the wild-type Pd1222 strain). Western blot analysis also confirmed that γ-subunit was not produced in either the cytoplasm or the periplasm (Fig. 5B). When the PdKO6 strain was transformed with expression plasmids for the long and short versions of γ-subunit (pKO30-ORF3-long and pKO30-ORF3-short, respectively), a marked difference was observed in the γ-subunit expression pattern (Fig. 5B); the γ-subunit protein was produced only in the PdKO6 cells transformed with pKO30-ORF3-long. Also, the QHNDH activity was partially restored in the cell-free extract of PdKO6 carrying pKO30-ORF3-long.

TABLE 1
Assignment of peaks from mass spectrometry analysis

The observed average mass is shown for [M+H]+ with S.D. (n = 5) for the processed and cytoplasmic γ-subunits, and the observed monoisotopic mass is shown for [M+H]+ with S.D. (n = 3) for the assigned Asp-N peptides. The calculated average mass is shown for [M+H]+ for the processed and cytoplasmic γ-subunits, and the calculated monoisotopic mass for [M+H]+ is shown for the assigned Asp-N peptides. PE, S-pyridylethylated; MSO, methionine sulfoxide; MSN, methionine sulfone.

| Experimental mass | Calculated mass | Modification | Sequence positions |
|-------------------|----------------|--------------|-------------------|
| Processed γ-subunit | 8,857.42 ± 5.95 | 8,857.57 | 3 Thiether, CTQ | 2–82 |
| Cytoplasmic γ-subunit | 12,423.36 ± 4.35 | 12,427.92 | 4 PE, 2 MSO | -28–82 |
| Assigned Asp-N peptide | | | |
| 1 | 746.86 | 746.32 | PE | 33–38 |
| 2 | 1,278.12 ± 0.80 | 1,277.58 | PE | 39–48 |
| 3 | 1,657.97 ± 0.40 | 1,657.74 | PE | 18–32 |
| 4 | 1,674.58 ± 0.30 | 1,673.73 | PE, MSO | 18–32 |
| 5 | 1,865.88 ± 0.31 | 1,864.96 | PE | 67–82 |
| 6 | 2,342.08 ± 0.48 | 2,341.03 | PE | 12–32 |
| 7 | 4,255.85 ± 0.64 | 4,255.10 | PE | 12–11 |
| 8 | 4,271.21 ± 0.04 | 4,271.09 | PE, MSO | 28–11 |
| 9 | 4,286.95 ± 0.71 | 4,287.09 | PE, 2 MSO | 28–11 |
| 10 | 4,300.97 ± 0.87 | 4,303.08 | PE, MSO, MSN | 28–11 |

* The number 1 Asp-N peptide was observed only once over the three time measurements.
ORF3-long (0.024 units/mg), although it corresponded only about 3% of the activity of the wild-type Pd1222 cells. It is likely that even if the expression of the γ-subunit is restored to the wild-type level by the introduced plasmid, the significantly reduced expression level of β-subunit in the PdKO6 mutant (about 6% of the wild-type by Western blotting, whereas that of α-subunit was 45% of wild-type) limits the active QHNDH complex formation. Because the β-subunit gene is encoded immediately 3′-downstream of the ORF3 gene, disruption of the ORF3 gene by the Km’ gene insertion may have decreased the expression of 3′-downstream gene products; the γ-subunit remaining probably without forming the QHNDH complex is detected in the cytoplasm (Fig. 5B). In contrast, the PdKO6 strain transformed with pKO30-ORF3-short produced neither the γ-subunit protein (Fig. 5B) nor the QHNDH activity in the cell-free extract (0.003 units/mg, comparable with that of the PdKO6 strain). Altogether, these findings show that the 28-residue longer form is an actual precursor of γ-subunit to be properly processed. Thus, similar to the α- and β-subunits, the γ-subunit also has a putative, though atypical signal sequence, likely functioning in the translocation into the periplasm. Alternatively, it may participate somehow in the processing of γ-subunit within the cytoplasm instead of serving as a signal peptide for periplasmic translocation.

It might be necessary to explain why an ~4-kDa mass difference between the unprocessed and mature γ-subunit polypeptides was not observed on SDS-PAGE in Fig. 2D and Fig. 5B. As reported previously for the crystal structure of QHNDH (5, 6) and also in the present paper (Fig. 6B), the mature γ-subunit has a mass of 8857 Da but behaves anomalously on SDS-PAGE to have an apparent mass of nearly 13 kDa, like the γ-subunit of P. putida enzyme that was also overestimated on SDS-PAGE to be about 20 kDa (3), presumably due to the highly cross-linked structure. On the other hand, the unprocessed γ-subunit migrates normally for a 12.4-kDa protein, resulting in the similar migration on SDS-PAGE with the mature γ-subunit (Figs. 2D and 5B).

**DISCUSSION**

The γ-subunit of QHNDH must undergo three types of posttranslational modification before functioning in the final destination, the periplasmic space of Gram-negative bacteria. One is the formation of the Cys37-Trp43 cross-bridge in the CTQ cofactor, the second is the introduction of a pair of oxygen atoms into the indole ring of Trp43, also in CTQ, and the third is the cross-linking of the side chains of 3 Cys residues (Cys7, Cys27, and Cys41) to the CB or Cy atoms of carboxylic acid side chains of Glu16, Asp33, and Asp49 (see Fig. 1C) (4–6). It is unknown whether these modifications of the γ-subunit occur before or after the formation of the αβγ heterotrimeric complex of QHNDH. It is also unknown whether the heterotrimeric complex is formed before or after the translocation of each subunit polypeptide into the periplasm independently or cooperatively. Furthermore, it is unknown whether the CTQ cofactor and/or the Cys-to-Asp/Glu cross-links are synthesized in a self-catalytic process or in a process depending on another enzyme or activating factor(s). Based on the results obtained in the present studies and also referring to those reported previously for the biogenesis of the analogous Trp residue-derived cofactor TTQ in MADH (25–30), we hypothesize that the Cys-to-Asp/Glu thioether cross-links are formed within the cytoplasm by the action of ORF2 protein on a nascent (or growing) polypeptide chain of the γ-subunit during or immediately after the translation before associating with other subunits, whereas the CTQ cofactor also contained in the γ-subunit is generated within the periplasm, possibly after forming a complex with the α-subunit, as discussed later.

There are structurally common features in the Cys-to-Asp/Glu thioether cross-links of the γ-subunit of QHNDH from both *P. denitrificans* (5) and *P. putida* (6). 1) As is obvious from the schematic structure shown in Fig. 1C, all Cys residues are N-terminal preceding the Asp or Glu cross-link partner in the γ-subunit polypeptide chain. 2) In addition, the cross-links are formed between a Cys and an Asp or Glu residue separated only by 5–8 residues, not between those separated by >10 residues; e.g. not between Cys7 and Asp33 or Cys27 and Asp49. 3) As identified in the crystal structures, all the methylene carbon atoms of Asp and Glu residues involved in the cross-linking have an S-configuration after cross-linking (5, 6). These features strongly suggest that the formation of the Cys-to-Asp/Glu thioether cross-links is not an arbitrary event but a strictly controlled chemical process occurring within the cells. To craft the cross-link structures with these common features, it is very likely that a protein is involved that scaffold a nascent polypeptide segment of the γ-subunit, containing a Cys residue and its Asp or Glu cross-link partner, and assists the sulfur atom of a Cys residue, attacking the activated methylene carbon atom of an Asp or Glu residue spatially only from one direction to form the cross-linked carbon atoms with S-configuration. Because the γ-subunit polypeptide without bearing any posttranslational modification is accumulated in the cytoplasm of the ORF2 gene-lacking PdKO4 mutant cells, as demonstrated here, it is highly conceivable that the ORF2 protein plays such a scaffolding role in the cross-link formation of the γ-subunit. It should be noted that there is no region that could function as a signal peptide in the deduced amino acid sequence of the ORF2 protein (4, 5), unlike the three subunits of QHNDH encoded in the same n-butylamine-utilizing operon. Indeed, the ORF2 protein was detected in the cytoplasm by Western blotting (Fig. 3C). Therefore, the ORF2 protein should work within the cytoplasm. Furthermore, site-specific mutations of invariant residues in the putative [Fe-S]–cluster and SAM binding motifs of ORF2 protein failed to rescue the QHNDH activity and growth of the PdKO4 strain (Fig. 4B). All these results support the hypothesis that the cytoplasmic ORF2 protein is involved in the thioether cross-link formation of the γ-subunit of QHNDH, serving not only as a scaffold protein but also as a direct activator as a Radical SAM protein.

Several proteins known as activating factors for other enzymes are included in the Radical SAM superfamily (12). For example, the pyruvate formate-lyase activating enzyme contains the [4Fe-4S] cluster and utilizes SAM to activate pyruvate formate-lyase by forming a peptidyl glycol radial (31). An enzyme called “activase” participates in the activation of anaerobic ribonucleotide reductase also through the glycol radical formation by using the [4Fe-4S] cluster and SAM (32). Another Radical SAM protein is involved in the posttranslational conversion of a Cys or Ser residue located in a conserved (C/S)XPXR motif in sulfatases into the catalytic residue, formylglycine, by a radical mechanism in which the 5′-deoxyadenosyl radical produced from SAM abstracts a hydrogen from the Ser/Cys side chain carbon atom for oxidation to the formyl group (33). Analogous to these Radical SAM proteins involved in enzyme activation, the putative [Fe-S]-cluster- and SAM-binding ORF2 protein presumably functions in activating the methylene carbon atoms of Asp and Glu residues in the γ-subunit of QHNDH for the formation of the Cys-to-Asp/Glu thioether cross-links by a radical mechanism. However, to conclude this, biochemical characterization by using the ORF2 protein purified in a native form containing an [Fe-S] cluster is essential, which is under way.

Although QHNDH has originally been identified only in two Gram-negative bacteria, *P. denitrificans* (1, 2) and *P. putida* (3), recent progress of bacterial genome projects has revealed the presence of a gene cluster homologous to QHNDH genes in several other bacteria, such as
Pseudomonas fluorescence, Azotobacter sp., and Novosphingobium aromaticivorans, indicating wide distribution of the QHNDH operon, which would generally function in the bacterial utilization of various amines. Amino acid sequences of the proteins corresponding to the ORF2 protein of *P. denitrificans*, found in those bacteria (*P. denitrificans*, GenBank™ accession number BAB78727; *P. putida*, GenBank™ accession number BAB72009; *P. fluorescens* PFO-1, GenBank™ accession number ABA74715; *Azotobacter sp.* EbN1, GenBank™ accession number CAI07355; *N. aromaticivorans* DSM 12444, GenBank™ accession number ZP_00872990), show 36–78% identities with the sequence 1.

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