C-terminal Periplasmic Domain of Escherichia coli Quinoprotein Glucose Dehydrogenase Transfers Electrons to Ubiquinone*  

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Membrane-bound quinoprotein glucose dehydrogenase (GDH) in Escherichia coli donates electrons directly to ubiquinone during the oxidation of D-glucose as a substrate, and these electrons are subsequently transferred to ubiquinol oxidase in the respiratory chain. To determine whether the specific ubiquinone-reacting site of GDH resides in the N-terminal transmembrane domain or in the large C-terminal periplasmic catalytic site of GDH, we constructed a fusion protein between the signal sequence of β-lactamase and cGDH. This truncated GDH was found to complement a GDH gene-disrupted strain in vivo. The signal sequence of the fused protein was shown to be cleaved off, and the remaining cGDH was shown to be recovered in the membrane fraction, suggesting that cGDH has a membrane-interacting site that is responsible for binding to membrane, like peripheral proteins. Kinetic analysis and reconstitution experiments revealed that cGDH has ubiquinone reductase activity nearly equivalent to that of the wild-type GDH. Thus, it is likely that the C-terminal periplasmic domain of GDH possesses a ubiquinone-reacting site and transfers electrons directly to ubiquinone.

Membrane-bound GDH1 in Escherichia coli is a PQQ-containing quinoprotein that catalyzes a direct oxidation of D-glucose to D-gluconate in the periplasm and concomitantly transfers electrons to ubiquinol oxidase through ubiquinone in the respiratory chain (1–3). GDH is an 88-kDa monomeric protein with five transmembrane segments at the N-terminal portion (residues 1–140), which ensure a strong anchorage of the protein to the inner membrane (4, 5). The remaining large C-terminal portion (residues 141–796) has a catalytic domain including PQQ (6, 7) and Ca2+ or Mg2+ -binding sites (8, 9) that is located in the periplasmic side. A model structure of GDH based on the x-ray crystallographic structure of the α-subunit of MDH in Methylobacterium extorquens has been proposed (10), and the putative structure of the PQQ-binding catalytic site has been further confirmed and characterized by mutagenic analysis of several amino acid residues around PQQ (11–15).

The ubiquinone-reacting site in GDH has also been analyzed. Friedrich et al. (16) proposed that the ubiquinone-reacting site may be located at the N-terminal transmembrane domain of Acinetobacter calcoaceticus GDH in which Arg-91 and Asp-93 may be involved in interaction with ubiquinone. The topological model of the N-terminal transmembrane domain of E. coli GDH has shown that the corresponding amino acid residues, Arg-93 and Asp-95, are located near the membrane surface of the periplasmic side (5). Furthermore, using depth-dependent fluorescent ubiquinone analogues, Miyoshi et al. (17) demonstrated that the ubiquinone-reduction site of GDH is located close to the membrane surface rather than in the hydrophobic interior. X-ray crystallographic structures of cytochrome bo in E. coli (18, 19) and cytochrome bc2 complex (Q, and Q, centers) in bovine heart mitochondria have recently been determined (20, 21), and it has been indicated that their ubiquinone-binding sites may be close to the membrane surface. Thus, it seems reasonable that the ubiquinone-reacting site of GDH is located near the membrane surface. However, in quinohemoprotein alcohol dehydrogenase of acetic acid bacteria despite the absence of a transmembrane domain, subunit II appears to be embedded in the cytoplasmic membrane and contain the ubiquinone-reduction site (22, 23). In addition, in the case of E. coli GDH, the mutations in the possible ubiquinone-reacting sites, Arg-93 and Asp-95, in the transmembrane domain have been shown to have no effect on the ubiquinone reductase activity.2 Thus, we cannot exclude the possibility that the C-terminal periplasmic domain of GDH, named cGDH, contains the ubiquinone-reacting site.

In efforts to clarify the role of the cGDH in reaction with ubiquinone, we constructed a fusion protein that was composed of the β-lactamase signal sequence (residues 1–25) and the cGDH (residues 142–796). The signal sequence was found to export the cGDH through the inner membrane to the periplasm, and the truncated GDH successfully complemented a GDH gene-disrupted strain in vivo and in vitro. In this paper, we also discuss the evolutionary relationship between GDH in E. coli and SLDH in Gluconobacter suboxydans or MDH in M. extorquens.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Hertfordshire, United Kingdom). Oligonucleotide primers were purchased from Sawady Technology (Tokyo, Japan). Q2

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1 The abbreviations used are: GDH, glucose dehydrogenase; cGDH, C-terminal periplasmic domain of GDH; PQQ, pyrroloquinoline quinone; MDH, methanol dehydrogenase; SLDH, sorbitol dehydrogenase; Q-2, ubiquinone-2; IPTG, isopropyl-β-D-thiogalactopyranoside; PMS, phenazine methosulfate; MOPS, 3-(N-morpholino)propanesulfonic acid; KPB, potassium phosphate buffer; MBP, maltose-binding protein; ORF, open reading frame; LB, L broth.
2 MD. Elias, M. Tanaka, M. Sakai, H. Toyama, K. Matsushita, O. Adachi, and M. Yamada, unpublished data.
was kindly provided by Eiizai Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade and obtained from commercial sources.

**Bacterial Strains, Plasmids, and Media**—The E. coli K-12 strains used in this study were PPA232 (ΔptsH ptsP crr::gal::Thn10), derived from the wild-type PB8 strain (9), and YU423 (PPA232 gcd::cm) (9). The plasmids pBLAGCD, pMC1396 (lacI::Tn10 [lacR amp’]), and pUCCGCD (amp’ gcd’l) (26). Plasmid pBLAGCD was constructed by the insertion of the DNA fragment encoding amino acid residues 1–25 of β-lactamase that was fused with the DNA fragment encoding residues 172–796 of GDH (see Fig. 1) into pTQ18.

E. coli was grown in LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl) or minimal medium (1.29% Na2HPO4, 0.3% KH2PO4, 0.05% NaCl, 0.1% NH4Cl, 0.025% MgSO4, 0.001% CaCl2, and 0.001% vitamin B12) or on agar plates. PQQ (200 μM), IPTG (0.1 mM), ampicillin (50 μg/ml), and 0.2% glucose or 0.4% glycerol as a carbon source were added as supplements to the LB or minimal medium as required.

**Construction of pBLAGCD Encoding a Bla-gDH Fusion Protein**—Conventional recombinant DNA techniques were applied (27). To produce a Bla-gDH fusion protein, the N-terminal membrane domain of GDH was maintained with the β-lactamase signal sequence as shown in Fig. 1A. A DNA fragment encoding the β-lactamase signal sequence (residues 1–25) was amplified by PCR with a set of primers, lacZ5′-TTCGTTGCGCCCGAACGCAAG-3′ and bla-sg1 5′-AAGCTTGCATGC-3′ containing BamHI and PstI sites of the vector pQ18I. The PCR products were recovered by XhoI and BamHI digestion of pMC1396 DNA as a template. A DNA fragment encoding the C-terminal periplasmic domain of GDH (residues 142–796) was amplified by PCR with a set of primers gcd-sg2 5′-CTCTTGATCCGCAGGAG-3′ and bla-sg1 5′-AAGCTTGCATGC-3′ containing BamHI and PstI sites of the vector pQ18I. The PCR products were recovered by BamHI digestion of pMC1396 DNA as a template. A DNA fragment encoding the C-terminal periplasmic domain of GDH was digested with BamHI and PstI, and the digested products were ligated into pBLAGCD generating pBLAGCD. The insertion was confirmed by restriction mapping and by DNA sequencing (28) using a thermo sequence cycle sequencing kit (Amer sham Biosciences, Inc.).

**Complementation Test of gDH to YU423 Strain**—For the complementation test in liquid medium, a single colony of PPA232-harboring pTQ18, YU423-harboring pBLAGCD, or YU423-harboring pTQ18 was inoculated into 2 ml of minimal medium supplemented with 0.4% glycerol and incubated for 48 h at 30°C while being shaken. The cells were diluted to a turbidity corresponding to an optical density at 600 nm (A600) of 1.0. 100 μl of the diluted preculture was then inoculated into 50 ml of minimal medium supplemented with 0.2% glucose, 200 mM PQQ, and 0.1 mM IPTG and incubated at 30°C. Cell growth was followed by measuring the turbidity at 660 nm. After the turbidity had reached 1, the cell suspension was centrifuged at 86,000 × g for 90 min. The supernatant was then centrifuged at 16,000 × g for 10 min to separate the supernatant and precipitate. The latter was resuspended in 10 mM KPB, pH 7.0, and treated by a French pressure cell press. In both samples, the activity of periplasmic β-lactamase as a control was measured as described previously (29), and 80 and 20% activities of β-lactamase were recovered in the supernatant and precipitate, respectively. Thus, the supernatant was used as the periplasmic fraction.

**Purification of Wild-type gDH and gDH**—YU423 harboring the wild-type GDH-encoding plasmid pUCCGCD was grown in LB medium for 30 h at 25°C. The cells were harvested by centrifugation and the membrane fraction according to the procedure described previously (5).

YU423 harboring pBLAGCD was grown at 25°C in LB medium supplemented with 0.2% glucose and 200 nM PQQ. IPTG was added at the final concentration of 0.1 mM into the culture when A600 had reached ~0.3 and the cells were grown for an additional 4 h. All purification steps were then carried out at 4°C. The membrane fraction prepared as described above was washed with 1× KCl by stirring for 30 min and then centrifuged at 86,000 × g for 90 min. The pellet was homogenized to a final protein concentration of 10 mg/ml in 10 mM KPB, pH 7.0, containing 1 mM MgCl2 and stirred in the presence of 0.3% Triton X-100 (w/v), 100 mM PQQ, and 100 mM KCl for 30 min for solubilization of the enzyme from the membrane fraction. The suspension was centrifuged at 86,000 × g for 90 min, and the supernatant obtained was dialyzed against the same buffer containing 0.1% Triton X-100. The dialyzed was applied onto a DEAE-Toyopearl column (1 ml bed volume/10 mg of protein) equilibrated with 10 mM KPB, pH 7.0, containing 1% Triton X-100. The incubated at 25°C for 90 min in 0.3% KH2PO4, 0.05% NaCl, 0.1% NH4Cl, 0.025% MgSO4, 0.001% CaCl2, and 0.001% vitamin B12 on agar plates. PQQ (200 μM), IPTG (0.1 mM), ampicillin (50 μg/ml), and 0.2% glucose or 0.4% glycerol as a carbon source were added as supplements to the LB or minimal medium as required.

**Preparation of Microbial and Periplasmic Fractions**—For preparation of the membrane fraction, cells grown as described in the following enzyme purification procedure were harvested by centrifugation and quickly chilled on ice. All of the subsequent steps were carried out at 4°C. The cells were washed twice with 0.85% NaCl and suspended in 10 mM KPB, pH 7.0. The membrane fraction was then prepared as described previously (5) and homogenized to a final protein concentration of 10 mg/ml in the same buffer containing 1 mM MgCl2. For preparation of the periplasmic fraction, cells grown as described in the following enzyme purification procedure were washed with 10 mM Tris-HCl, pH 7.0, containing 30 mM NaCl. The cells were resuspended in 33 mM Tris-HCl, pH 7.0, containing 0.1 mM EDTA and 20% sucrose. The suspension was stored at room temperature for 10 min. After centrifugation, the pellet was resuspended in 20 ml of ice-chilled 0.5 mM MgCl2 and gently stirred for 10 min in an ice bath. The suspension was then centrifuged at 16,000 × g for 10 min to separate the supernatant and precipitate. The latter was resuspended in 10 mM KPB, pH 7.0, and treated by a French pressure cell press. In both samples, the activity of periplasmic β-lactamase as a control was measured as described previously (29), and 80 and 20% activities of β-lactamase were recovered in the supernatant and precipitate, respectively. Thus, the supernatant was used as the periplasmic fraction.

**Electron Transfer from Glucose Dehydrogenase to Ubiquinone**

Electron transfer from glucose dehydrogenase to ubiquinone (electron mediator and acceptor, respectively) as described previously (13, 32). Glucose oxidase activity of membrane fractions (coupling ability of GDH to an electron transport chain in the membrane) was determined using an oxygen electrode as described previously (13, 32). Q2 reductase activity was measured spectrophotometrically as described previously (13). One unit of PMS reductase or Q2-reductase activity is
defined as 1 μmol of dichloroindophenol or Q-2, respectively, reduced/min, both of which correspond to 1 μmol of glucose oxidized/min. One unit of glucose oxidase activity is defined as 1 μmol of oxygen consumed/min, which is also equivalent to 1 μmol of glucose oxidized/min. The K_m value was estimated on the basis of a Lineweaver-Burk plot.

Reconstitution of Purified Wild-type GDH or cGDH with the YU423 Membrane Fraction—The YU423 membrane fraction was suspended in 10 mM KPB, pH 7.0, at a final protein concentration of ~30 mg/ml. For the reconstitution experiments, Triton X-100 in the purified wild-type GDH (15 units/50 μl) was replaced with 1% Triton X-100, and GDH and cGDH enzyme solution was reconstituted with 1% Triton X-100 in the purified wild-type GDH (15 units/50 μl) at a final protein concentration of 1 mg/ml. After the reconstitution experiments, Triton X-100 in the purified wild-type GDH (15 units/50 μl) was removed during purification. Although two other minor bands of approximately 45 kDa still remained after ceramic hydroxyapatite column chromatography, we used this sample for enzymatic analysis or reconstitution experiments.

Because cGDH was purified from the membrane fraction, it was unclear whether the signal sequence was cleaved from cGDH or not. The N-terminal amino acid residues were thus sequenced with the 75-kDa protein band excised from the blotted membrane. The sequence of the first nine amino acids obtained was His-Pro-Asp-Pro-Glu-Ile-Asn-Gly, which fully corresponds to the sequence of the fusion protein shortly after the β-lactamase signal cleavage site as shown in Fig. 1. Therefore, it is likely that the β-lactamase signal sequence of the fusion protein is proteolytically cleaved during secretion, and that the cleaved cGDH binds to the membrane without the signal sequence.

Characteristics of cGDH—To characterize cGDH and compare it with the wild-type GDH, kinetic analysis was performed with membrane fractions and both purified enzymes (Tables II and III). Glucose oxidase activity in the membrane fraction reflects the ability of the intermolecular electron transfer from cGDH to membrane ubiquinone and finally to terminal oxidase. Surprisingly, we found that the membrane fraction prepared from YU423-harboring pBLAGCD possessed significant glucose oxidase activity as well as PMS reductase activity. On one hand, these relative activities are comparable to those of PPA322 containing the wild-type GDH gene on the genome and harboring the vector plasmid pTTQ18, which are calculated based on their relative GDH contents in the membrane fraction estimated by Western blot analysis (Fig. 2A). On the other hand, no detectable activity was observed in a GDH gene-disrupted strain, YU423, harboring pTTQ18. Therefore, it is likely that cGDH is able to transfer electrons to ubiquinone. Notably, cGDH contains a catalytic domain including PQQ- and Ca^2+_– binding sites (6–9). We used the PMS reductase and Q-2 reductase activities in purified cGDH and wild-type GDH (Table III). The purified cGDH was found to have almost equivalent activities of both reductases to those of the wild-type GDH. This outcome is because the purity of cGDH and that of wild-type GDH was 50% (Fig. 2B) and 95%, respectively. On the basis of these activities after purification, approximately half of cGDH was assumed to be inactive in membrane fraction, which was removed during purification.
Electron Transfer from Glucose Dehydrogenase to Ubiquinone

Fig. 2. Cellular localization of cGDH (A) and SDS-12% polyacrylamide gel electrophoresis of purified cGDH sample (B).

TABLE I

| Purification of cGDH from the membrane of YU432 harboring pBLAGCD |
|---------------------------------------------------------------|
| Steps | Protein | PMS reductase activity of cGDH | Recovery | Purification fold |
|-------|---------|-------------------------------|----------|-----------------|
|       | mg      | units | units/mg | %      |                 |
| Membrane fraction | 1000 | 100 | 0.10 | 100 | 1 |
| Washed membrane | 850 | 100 | 0.11 | 100 | 1 |
| Triton X-100 extract | 110 | 70 | 0.63 | 70 | 7 |
| DEAE-Toyopearl | 8.0 | 50 | 6.3 | 50 | 62 |
| Ceramic hydroxyapatite | 0.13 | 20 | 150.0 | 20 | 1500 |

a PMS reductase activity was measured after holo-enzyme formation as described under “Experimental Procedures.”

b Membrane fraction was washed with 1 M KCl.

TABLE II

| PMS reductase and glucose oxidase activities of cGDH and wild-type GDH in membrane fractions and their K_m values for PQQ and glucose |
|--------------------------------------------------------------------------------------------------------------------------------|
| Strains | PMS reductase activity | Glucose oxidase activity | Relative GDH amount in membrane | Relative PMS reductase activity | Relative glucose oxidase activity | K_mPQQ | K_mGlc |
|---------|------------------------|--------------------------|---------------------------------|-----------------------------|-------------------------------|--------|--------|
|         | milliunits/mg | % | milliunits/mg (%) | % | nm | nm |
| PPA322/pTTQ18 | 250 | 110 | 100 | 250 (100) | 100 (100) | 110 | 0.9 |
| YU423/pBLAGCD | 100 | 80 | 80 | 125 (50) | 63 (63) | 300 | 1.4 |
| YU423/pTTQ18 | <0.01 | <0.01 | ND | ND | ND | ND | ND |

a Glucose oxidase respiratory chain activity.
b The relative amount of GDH protein in the membrane fractions was estimated from the results of the Western blot shown in Fig. 2A and expressed as a percentage of that from the wild type.
c Values of PMS reductase and glucose oxidase activities were corrected by the relative amount of GDH protein in the membrane fractions. Values in parentheses are percentages of activity of the wild type.

This is because the relative PMS reductase and glucose oxidase activities of cGDH in membrane fraction was 50 and 63%, respectively, compared with those of wild-type GDH (Table II).

To further examine whether the deletion of the N-terminal hydrophobic domain had any effect on the affinity for PQQ, glucose, or Q-2, the K_m values of cGDH were compared with those of wild-type GDH. The K_m values for PQQ and glucose of cGDH in the membrane fractions were found to be nearly the same as those estimated in wild-type GDH (Table II). The K_m values for PQQ, glucose, and Q-2 of the purified cGDH were also found to be almost equivalent to those of the purified wild-type GDH (Table III). Thus, the deletion of the N-terminal hydrophobic domain appears to have no significant effect on the affinity for PQQ, glucose, or Q-2, and cGDH seems to possess the ubiquinone-reduction site in its sequence. Furthermore, we examined the substrate specificity of the purified

TABLE III

| PMS and Q-2 reductase activities of purified cGDH and wild-type GDH and their K_m values for PQQ, Q-2, and glucose |
|--------------------------------------------------------------------------------------------------------------------------------|
| Purified enzymes | PMS reductase activity | Q-2 reductase activity | K_mPQQ | K_mGlc | K_mQ-2 |
| Wild-type GDH | 300 | 40 | 110 | 0.95 | 25 |
| cGDH | 150 | 20 | 120 | 0.98 | 30 |
Electron Transfer from Glucose Dehydrogenase to Ubiquinone

Regeneration of Glucose Oxidase Activity in the YU423 Membrane with Purified cGDH—To examine whether cGDH can interact with the cytoplasmic membrane and whether it can donate electrons directly to the intrinsic ubiquinone, purified cGDH or wild-type GDH was mixed with the YU423 membrane fraction in the presence of β-octyl-d-glucopyranoside. After removing the detergent from the samples by dialysis, membranes were collected by ultracentrifugation. As seen in Table IV, 55 and 60% activities of PMS reductase were recovered in cGDH-reconstituted and wild-type GDH-reconstituted membranes, respectively. Moreover, PMS reductase and glucose oxidase activities in the cGDH-reconstituted membrane were found to be comparable to those in the wild-type GDH-reconstituted membrane. These results suggest that cGDH interacts with the cytoplasmic membrane similar to the wild-type GDH and is able to donate electrons directly to ubiquinone.

**DISCUSSION**

Ubiquinone-reacting Site of GDH—The following findings obtained in this study clearly indicate that cGDH is bound to the cytoplasmic membrane. First, the signal sequence of β-lactamase was found to be absent from purified cGDH. Second, cGDH was recovered in the membrane fraction (Fig. 2A). Third, Triton X-100 was required for solubilization of the protein from the membrane fraction. Finally, cGDH is functionally reconstituted into the membrane fraction by the octylglucoside dialysis method. From these findings, it is believed that cGDH may have some amphiphilic segment responsible for binding to the membrane. The binding mode appears to be similar to those of peripheral proteins because no membrane-spanning segment exists in cGDH. GDH has such transmembrane segments in the N-terminal portion but not in the C-terminal portion (5), i.e. cGDH. This idea of cGDH being a peripheral protein was supported by results of experiments with a mild base. Treatment with 0.25 M NaOH caused a release of 60–70% of cGDH protein from the membrane fraction, whereas the same treatment could not release the wild-type GDH protein.

cGDH was found to show glucose oxidase activity equivalent to that of wild-type GDH in membrane fractions (Table II) as well as in reconstituted membranes (Table IV). Moreover, purified cGDH showed a significant Q-2 reductase activity, and the removal of the N-terminal hydrophobic portion had no influence on the affinity for Q-2. Judging from these findings, it seems that the ubiquinone-reacting site resides in the C-terminal periplasmic domain of GDH and that the site is close to the membrane surface, as supported by the evidence provided by Miyoshi et al. (17). Likewise, alcohol dehydrogenase of acetic acid bacteria may have a ubiquinone-reacting site in the peripheral cytochrome subunit (22). Originally, the ubiquinone-reacting site was proposed to be located in the N-terminal hydrophobic domain, especially Arg-91 and Asp-93, of A. calcoaceticus GDH based on the sequence homology to that of mitochondrial NADH (16). However, mutants R93A, R93D, D95A, and D95N of Arg-93 and Asp-95 in A. calcoaceticus GDH based on the sequence homology to that of mitochondrial NADH (16). However, mutants R93A, R93D, D95A, and D95N of Arg-93 and Asp-95 in A. calcoaceticus GDH showed no significant effect on PMS reductase and glucose oxidase activities and also on affinity for Q-2. Therefore, the possible involvement of Arg-93 and Asp-95 in interaction with ubiquinone was excluded. Thus, the ubiquinone-reacting site of GDH...
**Fig. 4.** Schematic representation of amino acid sequence homology between GDH and SLDH or MDH. GDH is a single protein (796 amino acids) with five transmembrane segments (dotted boxes) at its N terminus. SLDH (GenBank™ accession number E32232) seems to be encoded by two ORFs; the small one encodes a protein (120 amino acids) containing a signal sequence (SS). The MDH α-subunit consists of 626 amino acids including a signal sequence. Stripped boxes in the large protein of SLDH and in cGDH represent segments including putative membrane-binding and ubiquinone-reacting sites. Numbers indicated are the amino acid residues. Percentages show similarities between the small protein of SLDH and the first four N-terminal transmembrane segments of GDH between the C-terminal domain (residues 230–867) of the large protein of GDH and the C-terminal domain (residues 170–796) of GDH and between the C-terminal domain (residues 121–495 and 575–796) of GDH and the entire MDH α-subunit.

These proteins. We propose that the MDH α-subunit is significantly more similar to the common evolutionary origin than is GDH or SLDH, and that the common primordial protein had acquired a ubiquinone-binding site and a subunit containing four transmembrane segments as in SLDH, which had then fused to become a single protein like GDH.

At present, with the exception of the likelihood that the N-terminal transmembrane domain ensures a strong anchorage of GDH to the inner membrane since it is likely that the large C-terminal periplasmic domain possesses the catalytic site and ubiquinone-reacting site, which thus donates electrons directly to membrane ubiquinone, the role of the N-terminal domain in the electron transfer from glucose to ubiquinone is not clear. Considering the existence of multiple transmembrane segments in GDH, the hydrophobic domain may interact with the C-terminal domain and therefore somehow control its activity.

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