Functions of Amino Acid Residues in the Active Site of *Escherichia coli* Pyrroloquinoline Quinone-Containing Quinoprotein Glucose Dehydrogenase*

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Several mutants of quinoprotein glucose dehydrogenase (GDH) in *Escherichia coli*, located around its cofactor pyrroloquinoline quinone (PQQ), were constructed by site-specific mutagenesis and characterized by enzymatic and kinetic analyses. Of these, critical mutants were further characterized after purification or by different amino acid substitutions. H262A mutant showed reduced affinities both for glucose and PQQ without significant effect on glucose oxidase activity, indicating that His-262 occurs very close to PQQ and glucose, but is not the electron acceptor from PQQH$_2$. W404A and W404F showed pronounced reductions of affinity for PQQ, and the latter rather than the former had equivalent glucose oxidase activity to the wild type, suggesting that Trp-404 may be a support for PQQ and important for the positioning of PQQ. D466N, D466E, and K493A showed very low glucose oxidase activities without influence on the affinity for PQQ. Judging from the enzymatic activities of D466A and K493A, as well as their absorption spectra of PQQ during glucose oxidation, we conclude that Asp-466 initiates glucose oxidation reaction by abstraction of a proton from glucose and Lys-493 is involved in electron transfer from PQQH$_2$.

PQQ$^1$ is a non-covalently bound prosthetic group of most quinoprotein dehydrogenases in Gram-negative bacteria, which are involved in the oxidation of alcohols or aldoxone sugars in their periplasm. Membrane-bound quinoprotein GDH of *Escherichia coli* catalyzes oxidation of the C-1 hydroxyl group of the pyranose form of D-glucose to D-glucono-δ-lactone, which is spontaneously converted to D-gluconate, and concomitantly transfers electrons to ubiquinol via ubiquinone in the respiratory chain. Topological analysis revealed that the monomeric GDH possesses five trans-membrane segments at the N-terminal portion (residues 1–154), which ensure strong anchorage of the protein in the inner membrane. The remaining C-terminal portion (residues 155–796) occurs at the periplasmic side of the membrane. This portion is assumed to have a catalytic domain including PQQ (5, 6) and Ca$^{2+}$ or Mg$^{2+}$ binding sites (7, 8). Moreover, GDH of *E. coli* occurs as an apoenzyme (7, 8), and the exogenous addition of PQQ with the divalent cation leads to formation of the active enzyme (9).

Three-dimensional structures of MDHs from three different bacteria have been determined by x-ray crystallography (10–12), which reveals that the $\alpha$ subunit is a superbarrel made up of eight topologically identical four stranded anti-parallel $\beta$ sheets, being arranged with radial symmetry like the blades of a propeller. PQQ is tightly stacked within a chamber of the active site in the $\alpha$ subunit, and Ca$^{2+}$ helps PQQ to be maintained in the correct configuration. Amino acid residues interacting with PQQ and Ca$^{2+}$ are dispersed in the whole $\alpha$ subunit. Alignment of the PQQ-binding proteins or subunits among quinoprotein dehydrogenases reveals that the periplasmic domain of GDH in *E. coli* has 26% sequence similarity to the $\alpha$ subunit of MDH in *Methylobacterium extorquens* (13). On the basis of the superbarrel structure of the $\alpha$ subunit of MDH, a model structure of GDH was proposed except for its N-terminal trans-membrane domain and several unique segments lacking in MDH (13). The proposed model depicted the possible amino acid residues interacting with PQQ in the catalytic domain of GDH (Fig. 1). A key feature of PQQ structure is the *ortho*-quinone at the C-4 and C-5 positions, which becomes reduced to the quinol during catalysis (14, 15). In the oxidized state, the C-5 carbonyl is very reactive toward nucleophiles such as alcohols, ammonia, amines, cyanide, and amino acids (15, 16). The PQQ in GDH is proposed to be sandwiched between Trp-404 and His-262 by analogy with the MDH structure in *M. extorquens* (13), where PQQ is tightly stacked between Trp-243 and a novel disulfide ring composed of Cys-103 and Cys-104 (17). In addition to such vertical interactions with PQQ, there are many equatorial interactions between substituent groups of PQQ and amino acid residues in the model GDH, many of which are conserved in MDH (13). Among these residues, Lys-493 and Asn-607 are proposed to make hydrogen bonds with C-4 or C-5 carbonyl oxygen at the reactive site of PQQ, and Asp-466 is proposed to be involved in the initiation of glucose oxidation. Therefore, all the above residues are assumed to be important for the catalytic reaction of GDH. Some chemical approach (18) and mutagenic analyses (19–21) have been performed, but the evidence for the proposed catalytic domain structure and amino acid residues involved in the reaction is still limited.

To examine that assumption, we targeted the amino acid residues surrounding the reactive site of PQQ in the model. Several mutants were constructed by site-specific mutagenesis on the targeted residues and characterized by kinetic and spectral analyses. In comparison with other complex dehydro-
Quinoprotein Glucose Dehydrogenase Mutants

nases with several subunits in respiratory chains (22–26), a single protein GDH would be a good model to elucidate the molecular mechanism of catalytic reactions or intramolecular electron transfer. Here, we provide several lines of evidence for the molecular functions of the amino acid residues proposed in the active site of the model GDH. We also present a catalytic reaction mechanism of GDH based on that proposed for MDH (13, 27).

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction enzymes, T4 DNA ligase, and Taq DNA polymerase were purchased from Takara Shuzo (Kyoto, Japan). Oligonucleotide primers for site-specific mutagenesis were purchased from Sawady Technology (Tokyo, Japan). Q-2 was kindly supplied by Eizai Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade and obtained from commercial sources.

**Bacterial Strains and Plasmids**—The bacterial strains used in this study were derivatives of *E. coli* K-12. Their relevant genotype and plasmids are listed in Table I.

**Mutagenesis**—To construct all mutants of GDH, site-specific mutagenesis was carried out using the Mutan-Super Express Km kit (Takara Shuzo, Japan). Mutagenic primers used were: 5'-CTTCTTGCGCCGTAACCTG-3' (Takara Shuzo, Japan). Mutagenic primers used were: 5'-CTTCTTGCGCCGTAACCTG-3' for H262A, 5'-CAAAACTCGgGCAACGCACG-3' for W404A, 5'-CAAAACTCGCtGCAACGCACG-3' for W404F, 5'-CCGATTGGCgGtCCAAATGGC-3' for N607A, 5'-CTCGGCGGtCACAACGCAACG-3' for K493R, 5'-ACCTGTGGgAAgATGGATCTT-3' for D466A, 5'-ACCTGTGGgAAgATGGATCTT-3' for D466F, and 5'-ACCTGTGGgAAaATGGATCTT-3' for D466E.

PCR of 30 cycles for site-specific mutagenesis was performed by using a Takara PCR Thermal Cycler MP; each cycle consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 5 min. The PCR products were recovered by ethanol precipitation and introduced into MV1184. Kanamycin-resistance mutants were isolated, and mutation positions were confirmed by nucleotide sequencing (28) using the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech). Conventional recombinant DNA techniques (29) were used for exchanging the mutated regions with the corresponding in pUCGCD1 bearing the wild-type GDH gene. The recombinant mutant plasmids and their mutation positions were reconfirmed by restriction mapping and by DNA sequencing. Expression of GDH mutants was examined by SDS-polyacrylamide gel electrophoresis, followed by Western blotting using a polyclonal antibody raised against *E. coli* GDH as described previously (30).

**Preparation of Membrane Fractions and Purification of Mutant GDHs**—Cells harboring the wild-type plasmid, pUCGCD1, or mutant plasmids were grown in LB (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl) medium containing ampicillin (100 μg/ml) for 14 h at 37 °C. Membrane fractions were then prepared according to the procedure described previously (4). Critical mutant GDHs as well as the wild-type GDH were purified according to the procedure described (4, 19). For W404A mutant, all purification steps were performed in the presence of 0.1 μM PQQ to stabilize enzyme activity. Purified enzymes were analyzed by SDS-polyacrylamide gel electrophoresis to confirm their homogeneity.

**Enzyme Assay and Analytical Procedures**—PMS reductase activity and glucose oxidase activity (glucose oxidase respiratory chain activity) were measured as described (4, 31) except that instead of 10 mM KPB (pH 7.0), 5 mM MOPS (pH 7.0) was used as the assay buffer for purified enzyme in Table III. Note that PMS reductase activity with 5 mM MOPS (pH 7.0) was found to be about 2 times higher than that with 10 mM KPB (pH 7.0), reported previously (19). Q-2 reductase activity was measured at 25 °C by the addition of 33 mM glucose and 30 μM Q-2 in 5 mM MOPS (pH 7.0) after 20 min preincubation in the presence of 1 μM PQQ, 0.5 mM MgCl$_2$, and 0.025% Tween 20. One unit of PMS reductase and Q-2 reductase activities are defined as 1 μmol of 2,4-dichlorophenol indophenol and 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 microatom of oxygen consumed/min, which is equivalent to 1 μmol of glucose oxidized/min. $K_m$ values were estimated

| Strain or plasmid | Genotype and description | Reference or source |
|-------------------|--------------------------|---------------------|
| Strains           |                          |                     |
| YU423             | F8B Δ(ptsH ptsI crr) galP::Tn10 gcd::cm Δ(lac-proA), ara, rpsL, thi (680 lacZΔM15), Δ(srl-recA306)::Tn10 (terE)F[traD36, proAB', lacI', lacZΔM15] | Takara Shuzo (8)    |
| MV1184            |                          |                     |
| Plasmids          |                          |                     |
| pUCGCD1           | amp' gcd                 |                     |
| pUCGCDH262A       | gcd mutant H262A (CAC → GCG) | This work (19)     |
| pUCGCDW404A       | gcd mutant W404A (TGG → GCG) | This work (19)     |
| pUCGCDW404F       | gcd mutant W404F (TGG → TTT) | This work (19)     |
| pUCGCDN607A       | gcd mutant N607A (AAC → GCG) | This work (19)     |
| pUCGCDK493A       | gcd mutant K493A (AAA → GCA) | This work (19)     |
| pUCGCDK493R       | gcd mutant K493R (AAA → CGT) | This work (19)     |
| pUCGCDG466A       | gcd mutant D466A (GAC → GCC) | This work (19)     |
| pUCGCDG466N       | gcd mutant D466N (GAC → AAC) | This work (19)     |
| pUCGCDG466E       | gcd mutant D466E (GAC → GAA) | This work (19)     |
| pUCGCDG730N       | gcd mutant D730N (GAT → AAT) | This work (19)     |

**FIG. 1. Amino acid residues in the active site of the model GDH. A, the vertical interactions of PQQ with His-262 and Trp-404. B, the equatorial interactions of PQQ with Ca$^{2+}$ and several amino acid residues (reproduced, with permission, from Ref. 1). Amino acid substitutions were introduced to His-262, Trp-404, Asp-486, and Asp-666.**

| Table I  | Bacterial strains and plasmids |
|----------|--------------------------------|
| Strains  |                               |
| YU423    | FB8 Δ(ptsH ptsI crr) galP::Tn10 gcd::cm Δ(lac-proA), ara, rpsL, thi (680 lacZΔM15), Δ(srl-recA306)::Tn10 (terE)F[traD36, proAB', lacI', lacZΔM15] | Takara Shuzo (8) |
| MV1184   |                               |
| Plasmids |                                     |
| pUCGCD1  | amp' gcd                         |
| pUCGCDH262A | gcd mutant H262A (CAC → GCG) | This work (19) |
| pUCGCDW404A | gcd mutant W404A (TGG → GCG) | This work (19) |
| pUCGCDW404F | gcd mutant W404F (TGG → TTT) | This work (19) |
| pUCGCDN607A | gcd mutant N607A (AAC → GCG) | This work (19) |
| pUCGCDK493A | gcd mutant K493A (AAA → GCA) | This work (19) |
| pUCGCDK493R | gcd mutant K493R (AAA → CGT) | This work (19) |
| pUCGCDG466A | gcd mutant D466A (GAC → GCC) | This work (19) |
| pUCGCDG466N | gcd mutant D466N (GAC → AAC) | This work (19) |
| pUCGCDG466E | gcd mutant D466E (GAC → GAA) | This work (19) |
| pUCGCDG730N | gcd mutant D730N (GAT → AAT) | This work (19) |
on the basis of the Lineweaver-Burk plot. Protein content was determined according to the Dulley and Grieve method (32) using bovine serum albumin as a standard. Occurrence of the reduced form of PQQ during the catalytic reaction was examined by taking the absolute absorption spectra of purified apo-forms (4.5 μM each) of the wild-type and three mutant GDHs in 5 mM MOPS (pH 7.0) containing 0.1% alkylglucoside on a Shimadzu spectrophotometer (Multispec-1500). Spectra were then taken after holoenzyme formation, which was performed by incubation with 4.5 μM PQQ and 0.5 mM MgCl₂ for 20 min at 25 °C, and subsequently spectra were taken after the addition of 33 mM glucose to the holoenzyme.

RESULTS AND DISCUSSION

Stability and Characteristics of Mutant GDHs in Membrane Fractions—In order to examine the functions of the amino acid residues located close to the active site of the model GDH (Fig. 1), several substitutions were introduced into it by site-specific mutagenesis. The plasmid clones of the mutants and their mutation sites were shown in Table I. The stability and expression level of mutant GDH proteins were checked by Western blot analysis (Fig. 2). It revealed that contents of the mutant GDHs in the membrane fractions were 70–90% of that of the wild type except for D466A and W404A. The contents of the latter two mutants were about 10% and 40%, respectively, indicating that both GDH molecules may be unstable and presumably susceptible to intracellular proteolytic degradation.

Characteristics of the mutant GDHs were defined by the PMS reductase and glucose oxidase activities of the membrane fractions (Table II), which were calculated based on their relative GDH contents in the fractions estimated by Western blot analysis (Fig. 2). These activities and \( K_m \) values for PQQ and glucose were compared with those of the wild type. Moreover, some of the critical mutants were characterized after purification (Table III). Q-2 reductase activity was measured with the purified mutant GDHs, which may reflect the ability of intramolecular electron transfer. The \( K_m \) values for PQQ and glucose of the purified mutant GDHs were found to be nearly the same as those observed in the membrane fractions as shown in Table II. The K493A and D466E mutant GDHs showed very low Q-2 reductase activities compared with the wild type, which is consistent with the results of glucose oxidase activity observed in the membrane fractions.

Functions of His-262 and Trp-404—In the model GDH, PQQ was proposed to be sandwiched between Trp-404 on the lower side and His-262 on the upper side. As the disulfide ring in MDH of *M. extorquens* was proposed to play a role in electron transfer reaction (17), His-262 in GDH, being at the corresponding position to the ring, was postulated to act as an electron acceptor. The H262A mutation was found to have no significant effect on PMS reductase and glucose oxidase activities, but its affinities for glucose and PQQ were decreased about 11- and 8-fold, respectively, compared with those of the wild-type GDH in the membrane fractions (Table II). These results suggest that His-262 is not an electron acceptor from PQQH₂, but it may be located very close to PQQ and glucose molecules at the active site. Recently, a similar conclusion has been drawn for the H262Y mutant by Cozier et al. (21).

The W404A showed a pronounced reduction of affinity for PQQ and very low PMS reductase and glucose oxidase activities compared with the wild type (Table II). The lower activity is probably because the alanine molecule may be too small as a

![Fig. 2. Western blot analysis of the mutant GDHs obtained by site-specific mutagenesis.](image)

The membrane fractions from YU423 containing pUCGCD1 or pUCGCD mutants, which were used for estimating enzyme activities as shown in Table II, were subjected to a SDS-10% polyacrylamide gel electrophoresis and transferred to the blot membrane. The wild-type and mutant GDHs were visualized using a polyclonal antibody against *E. coli* GDH as described previously (30). The relative amount of GDH proteins were densitometrically estimated by using Bio-Rad Molecular Imager. YU423 containing pUCGCD1 or pUC118 (19) were used as a positive and negative control, respectively. Lanes 1–7 and 8–13 represent the membrane fractions from the negative control (30 μg), the positive control (wild type, 5 μg), the positive control (2.5 μg), H262A (5 μg), W404A (10 μg), W404F (5 μg), K493A (6 μg), D466N (6 μg), D466E (5 μg), D466A (10 μg), N607A (8 μg), K493R (5 μg), and the positive control (5 μg), respectively.

**Table II**

| Mutant | PMS reductase activity | Glucose oxidase activity | Relative GDH amount in membrane | Relative PMS reductase activity | Relative glucose oxidase activity | \( K_m \) | \( K_m \) |
|--------|------------------------|--------------------------|-------------------------------|-------------------------------|-------------------------------|---------|---------|
|        | milliunits/mg          | %                        |                               |                               |                               |         |         |
| Wild type | 12,000                | 100                      |                               |                               |                               | 2100    | 110     |
| H262A | 3900                  | 90                      |                               |                               |                               | 880     | 90      |
| W404A | 1.2                   | 30                      |                               |                               |                               | 3 (0.03)| 6000    |
| W404F | 140                   | 510                     |                               |                               |                               | 570     | 20,000 |
| N607A | 3600                  | 690                     |                               |                               |                               | 770     | 220     |
| K493A | 280                   | 6                       |                               |                               |                               | 8.6     | 50      |
| K493R | 85                    | 1                       |                               |                               |                               | 120     | 1.4     |
| D466E | 32                    | 24                      |                               |                               |                               | 40 (0.3)| 140     |
| D466N | 140                   | 18                      |                               |                               |                               | 200     | 140     |

*Glucose oxidase respiratory chain activity.*

*The relative amount of GDH protein in the membrane fractions was estimated from the results of Western blot shown in Fig. 2 and expressed as a percentage of that from the wild type.*

*Values of PMS reductase and 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.*

**TABLE II**

**PMS reductase and glucose oxidase activities of mutant GDHs in membrane fractions and their \( K_m \) values for PQQ and glucose**

**PMS reductase activity** was measured in the presence of 33 mM glucose, 0.5 mM MgCl₂, 1 μM PQQ, and 8 mM NaN₃ except that 10 μM PQQ was added at a final concentration for W404A, and 30 μM PQQ was added for W404F and K493R mutants. The preincubation requiring for holo-enzyme formation was performed at 25 °C for 20 min. Glucose oxidase activity was measured at 25 °C by the addition of 10 mM glucose after 20 min of preincubation in the presence of 1 μM PQQ and 0.5 mM MgCl₂, except that 10 μM PQQ was added at a final concentration for W404A, and 30 μM PQQ was added for W404F and K493R mutants. \( K_m \) values were estimated by measuring PMS reductase activity. Reported values are the averages of two to three independent experiments performed in triplicate.

| Mutant | PMS reductase activity | Glucose oxidase activity | Relative GDH amount in membrane | Relative PMS reductase activity | Relative glucose oxidase activity | \( K_m \) | \( K_m \) |
|--------|------------------------|--------------------------|-------------------------------|-------------------------------|-------------------------------|---------|---------|
|        | milliunits/mg          | %                        |                               |                               |                               |         |         |
| Wild type | 12,000                | 100                      |                               |                               |                               | 2100    | 110     |
| H262A | 3900                  | 90                      |                               |                               |                               | 880     | 90      |
| W404A | 1.2                   | 30                      |                               |                               |                               | 3 (0.03)| 6000    |
| W404F | 140                   | 510                     |                               |                               |                               | 570     | 20,000 |
| N607A | 3600                  | 690                     |                               |                               |                               | 770     | 220     |
| K493A | 280                   | 6                       |                               |                               |                               | 8.6     | 50      |
| K493R | 85                    | 1                       |                               |                               |                               | 120     | 1.4     |
| D466E | 32                    | 24                      |                               |                               |                               | 40 (0.3)| 140     |
| D466N | 140                   | 18                      |                               |                               |                               | 200     | 140     |

**TABLE III**

**Q-2 reductase activity measurements of mutant GDHs**

**Q-2 reductase activity** was measured in the presence of 8 mM NaN₃, 0.5 mM MgCl₂, 1 μM PQQ, 10 μM PMS, and 8 mM NaN₃ except that 1 μM PQQ was added at a final concentration for W404A, and 30 μM PQQ was added for W404F and K493R mutants. The preincubation requiring for holo-enzyme formation was performed at 25 °C for 20 min. Glucose oxidase activity was measured at 25 °C by the addition of 10 mM glucose after 20 min of preincubation in the presence of 1 μM PQQ and 0.5 mM MgCl₂, except that 10 μM PQQ was added at a final concentration for W404A, and 30 μM PQQ was added for W404F and K493R mutants. \( K_m \) values were estimated by measuring PMS reductase activity. Reported values are the averages of two to three independent experiments performed in triplicate.
lower planer support for PQQ. We thus constructed and tested another mutant, W404F. This mutant was found to have relatively higher activities of PMS reductase and glucose oxidase than W404A, although it showed extremely low affinity for PQQ (Table II). The substitution from Trp to Ala at position 404 may cause a local structural change, which influences the accessibility of PQQ or the positioning of PQQ in the catalytic site. Since W404F had nearly the equivalent glucose oxidase activity to that of the wild type, the Phe residue may be exchangeable with the Trp residue at least as a support for PQQ. Therefore, Trp-404 is assumed to be very close to PQQ and to confer a support for PQQ, which may be important for the positioning of PQQ as proposed previously (13). Notably, W404F was found to have very weak activity of PMS reductase but still retained glucose oxidase activity equivalent to that of the wild type (Table II). It is likely that somehow this mutation has weakened the electron transfer to PMS.

**Amino Acid Residues Close to the C-4 and C-5 Carbonyls of PQQ**—There are many equatorial interactions between subunit groups of PQQ and amino acid residues in the model GDH (13). In the active site of GDH, the PQQ ligation to Ca$^{2+}$ or Mg$^{2+}$ may be nearly the same as in MDH (18), but the ligation to the protein would be different from that in MDH. It was proposed that Asn-607 is equatorially interacting with C-4 and Lys-493 interacting with C-4 and C-5 of PQQ, and Asp-466 is a possible residue involved in the initial proton abstraction reaction from glucose (13). In order to clarify the catalytic reaction mechanism, substitutions were performed on these three residues, Asn-607, Lys-493, and Asp-466 of GDH.

The model GDH showed that the amide group of Asn-607 makes a hydrogen bond interaction with the C-4 carboxyl of PQQ (13). However, the N607A mutant showed only slightly reduced activities of PMS reductase and glucose oxidase without significant effect on the affinity for PQQ (Table II). Therefore, it is unlikely that Asn at the position 607 is a crucial residue for the catalytic reaction or the stabilization of PQQ in the active site.

The K493A and K493R mutants were found to have very low activities of both PMS reductase and glucose oxidase in the membrane fractions compared with the wild type (Table II), and purified K493A also showed very low Q-2 reductase activity (Table III). K493A seemed to have no effect on the affinity for PQQ but K493R showed a pronounced reduction of affinity for PQQ (Table II). These results suggest that the positive charge of Lys may be crucial for reactivity, but the alteration from Lys to Arg at position 493 may cause a local structural change, resulting in extremely low glucose oxidase activity as well as low affinity for PQQ. Since Arg occurs in MDH at the corresponding position to Lys-493 in GDH, the distance between Lys-493 and C-4 and/or C-5 carbonyl oxygen might be important.

**FIG. 3. Absolute absorption spectra of purified wild-type and mutant GDHs from E. coli**. The absorption spectrum was taken of 4.5 μM purified GDHs in 5 mM MOPS (pH 7.0) containing 0.1% alkylglucoside (Apo-GDH spectra). Holo-enzyme formation was performed by the addition of 4.5 μM PQQ and 0.5 mM MgCl$_2$ for 20 min at 25 °C. Spectra were taken of holomerized enzymes (+ PQQ spectra) and subsequently after the addition of 33 mM glucose, in which those at 10 s, 30 min, and 60 min are only shown (+ PQQ + Glu spectra). PQQ reduction abilities during glucose oxidation of mutant GDHs were compared with that of the wild type by their spectral changes in between 300 and 350 nm. Similar changes of absolute spectra were reported with membrane-bound apo-GDH from *Acinetobacter calcoaceticus* after adding PQQ and PQQ + substrate (37).

Asp-466 was replaced with Asn or Glu. The resultant D466N and D466E were shown to have very low PMS reductase and glucose oxidase activities in the membrane fractions without influences on the affinity for PQQ (Table II). Purified D466E also showed extremely low Q-2 reductase activity, but almost no effect on the affinity for PQQ (Table III). In addition, both D466N and D466E exhibited decreased affinities for glucose (Table II). These results suggest that Asp-466 may be located near the glucose molecule at the active site and important for the catalytic reaction. It is also suggested that Asp at position 466 is not exchangeable with Glu, presumably due to the re-

### Table III

| Mutant    | PMS reductase activity | Q-2 reductase activity | $K_m$ | $K_m$ |
|-----------|------------------------|------------------------|-------|-------|
| Wild type | 280 (100)              | 40 (100)               | 115   | 0.95  |
| K493A     | 3.0 (1.1)              | 0.03 (0.075)           | 53    | 1.1   |
| D466E     | 0.04 (0.14)            | ND                     | 62000 | 1.9   |
| K493A     | 3.0 (1.1)              | 0.03 (0.075)           | 53    | 1.1   |
| D466E     | 0.04 (0.14)            | ND                     | 62000 | 1.9   |
| D730N     | 9.0 (3.2)              | 4.0 (10)               | 120   | 0.80  |
| W404A     | 0.04 (0.14)            | ND                     | 62000 | 1.9   |

*Values in parentheses are percentages of activity of the wild type.

a Not determined.
involved in PQQH$_2$ oxidation by accepting abstraction of a proton and Lys-493 initiates the oxidation reaction of glucose by reduction of PQQH$_2$ and release of glucono-δ-lactone intermediate. The subsequent reaction leads to production of PQQH$_2$ and ubiquinone. Its function, however, is not clear because Asp-730 seems to be involved in the step between PQQH$_2$ and ubiquinone. Its function, however, is not clear because Asp-730 seems to be involved in the step between PQQH$_2$ and ubiquinone. Its function, however, is not clear because Asp-730 seems to be involved in the step between PQQH$_2$ and ubiquinone. Its function, however, is not clear because Asp-730 seems to be involved in the step between PQQH$_2$ and ubiquinone.

Judging from those results described above, at least Asp-466, Lys-493, and Asp-730 appeared to be crucial for a successive process of catalytic reactions from glucose oxidation to ubiquinone reduction. In order to further clarify their functions and positions in the catalytic cycle, we took the absolute absorption spectra of the purified apo- and holo-forms of the wild-type and mutant GDHs, and also followed changes of their spectra after the addition of glucose. As seen in Fig. 3, PQQ in the wild type could be almost completely reduced within 10 s after the addition of glucose to the holo-enzyme solution, while only 50% or less reduction of PQQ was found in D466E even 60 min after glucose addition. These results clearly suggest that Asp-466 is involved in a catalytic reaction before PQQ reduction while Lys-493 and Asp-730 perform their functions at the steps after PQQ reduction. The slow and gradual reduction of PQQ in the K493A or D730N mutants may be due to changes of the local structure near the reactive site of PQQ by these mutations. Alternatively, such mutations influenced the pK value of Asp-466 as postulated in MDH (33), which led to a decrease in the reaction rate.

Putative Catalytic Reaction Mechanism of GDH in E. coli—

Two types of mechanism have been proposed in the case of MDH for the initial proton-abstraction reaction from methanol: 1) a reaction mechanism via a covalent hemiketal intermediate and 2) a reaction mechanism by a hydride transfer (34, 35). At present, evidence supporting the former mechanism have been reported (34, 36).

Fig. 4 shows a putative catalytic reaction mechanism of E. coli GDH involving a hemiketal intermediate, where a negatively charged residue Asp-466 acts as a base, which initiates the oxidation reaction by abstraction of a proton from glucose. The proton abstraction leads to an oxyanion form of substrate, which attacks the electrophilic C-5 of PQQ, giving the hemiketal intermediate. The subsequent reaction leads to production of PQQH$_2$ and release of glucono-δ-lactone. After that, Lys-493 hydrogen-bonding with the C-4 and C-5 carbonyl oxygens of PQQ receives electrons as the first electron acceptor from PQQH$_2$, which allows PQQ to be back in the initial oxidized form.

On the other hand, this and previous data (19) indicated that mutants at position 730 reduced the GDH activity but D730N still retained the ability of PQQH$_2$ formation. Therefore, Asp-730 seems to be involved in the step between PQQH$_2$ and ubiquinone. Its function, however, is not clear because Asp-730 is not present in the active site of the model GDH proposed by Cozier and Anthony (13).

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