Amino Acid Residues Interacting with Both the Bound Quinone and Coenzyme, Pyrroloquinoline Quinone, in *Escherichia coli* Membrane-bound Glucose Dehydrogenase*1

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The *Escherichia coli* membrane-bound glucose dehydrogenase (mGDH) as the primary component of the respiratory chain possesses a tightly bound ubiquinone (UQ) flanking pyrroloquinoline quinone (PQQ) as a coenzyme. Several mutants for Asp-354, Asp-466, and Lys-493, located close to PQQ, that were constructed by site-specific mutagenesis were characterized by enzymatic, pulse radiolysis, and EPR analyses. These mutants retained almost no dehydrogenase activity or ability of PQQ reduction. CD and high pressure liquid chromatography analyses revealed that K493A, D466N, and D466E mutants showed no significant difference in molecular structure from that of the wild-type mGDH but showed remarkably reduced content of bound UQ. A radiolytically generated hydrated electron (e$_{aq}^{-}$) reacted with the bound UQ of the wild enzyme and K493R mutant to form a UQ neutral semiquinone with an absorption maximum at 420 nm. Subsequently, intramolecular electron transfer from the bound UQ semiquinone to PQQ occurred. In K493R, the rate of UQ to PQQ electron transfer is about 4-fold slower than that of the wild enzyme. With D354N and D466N mutants, on the other hand, transient species with an absorption maximum at 440 nm, a characteristic of the formation of a UQ anion radical, appeared in the reaction of e$_{aq}^{-}$, although the subsequent intramolecular electron transfer was hardly affected. This indicates that D354N and D466N are prevented from protonation of the UQ semiquinone radical. Moreover, EPR spectra showed that mutations on Asp-466 or Lys-493 residues changed the semiquinone state of bound UQ. Taken together, we reported here for the first time the existence of a semiquinone radical of bound UQ in purified mGDH and the difference in protonation of ubisemiquinone radical because of mutations in two different amino acid residues, located around PQQ. Furthermore, based on the present results and the spatial arrangement around PQQ, Asp-466 and Lys-493 are suggested to interact both with the bound UQ and PQQ in mGDH.

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The *Escherichia coli* membrane-bound glucose dehydrogenase (mGDH)$^2$ belongs to the quinoprotein family with PQQ as a coenzyme (1, 2), and it catalyzes d-glucose oxidation to d-glucuronate at the periplasmic side to transfer electrons to ubiquinol oxidase via UQ in the respiratory chain (3–5). Topological analysis revealed that mGDH consists of an N-terminal hydrophobic domain with five membrane-spanning segments and a large C-terminal domain residing in the periplasm, which contains PQQ and Ca$^{2+}$- or Mg$^{2+}$-binding sites in a superbarrel structure, conserved in quinoproteins (6–8). Although its tertiary structure has not been resolved, the arrangement of amino acid residues around PQQ has been modeled on the basis of the crystal structure of the quinoprotein methanol dehydrogenase (6) as depicted in Fig. 1. The arrangement has been confirmed by results of several experiments with site-directed amino acid substitutions (9–13). The orthoquinone portion of PQQ is a vital part for the catalytic reaction, to which Lys-493 hydrogen bonds in the model mGDH. Asp-466 is located close to the portion and functions in extracting a proton from glucose.

Several respiratory components, including primary dehydrogenases, have been found to possess bound UQ in their molecules (14–22), and in some of them, their local structures surrounding UQ have been disclosed. A single bound UQ was found in the crystal structures in succinate dehydrogenase (15) and from studies of mutants and Q-site inhibitors of the subunit of type I NADH dehydrogenase (16), but it is not clear whether a second site exists. The yeast mitochondrial type II NADH dehydrogenase has been demonstrated to have two UQ-binding sites (21), but the tertiary structure still remains to be resolved. The *E. coli* cytochrome *bd* ubiquinol oxidase has two UQ-binding sites with high (Q$_{b}$) and with low (Q$_{l}$) affinity for UQ (23, 24). The crucial amino acid residues interacting with UQ at Q$_{b}$ have been identified and shown to be conserved in cytochrome *bo* or *bd* ubiquinol oxidases (25). The UQ-binding site adopts a binding conformation similar to that of UQ-binding sites (Q$_{b}$ and Q$_{l}$) of the cytochrome *bc$_{1}$* complex in bovine mitochondria (26). Amino acid residues interacting with bound UQ in the *E. coli* cytochrome *bd* ubiquinol oxidase

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$^2$The abbreviations used are: mGDH, membrane-bound glucose dehydrogenase; PQQ, pyrroloquinoline quinone; UQ, ubiquinone; DM, N-dodecyl β-d-maltoside; MOPS, 3-(N-morpholino)propanesulfonic acid; KPB, potassium phosphate buffer; PMS, phenazine methosulfate; e$_{aq}^{-}$ hydrated electron; HPLC, high pressure liquid chromatography.
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![Diagram of amino acid residues interacting with bound quinone in mGDH](image)

(27) and in the E. coli DsbB related to the formation of disulfide bridges in secreted proteins (28) have also been reported. However, little information on the local structure or physiological function of bound UQ in primary components of respiratory chains has so far been available.

Although more than a dozen quinoprotein dehydrogenases have been discovered, little is known about their intramolecular electron transfer pathways. The E. coli mGDH has been demonstrated to have two UQ-binding sites, one (QI) for bound UQ and the other (QII) for bulk UQ (29), which is near the membrane surface rather than in the hydrophobic interior (30). In succession to the catalytic reaction, electrons from bound UQ and the other (QII) for bulk UQ (29), which is near the membrane surface rather than in the hydrophobic interior (30). In succession to the catalytic reaction, electrons from bound UQ and the other (QII) for bulk UQ (29), which is near the membrane surface rather than in the hydrophobic interior (30).

To construct the D354N mutant, site-specific mutagenesis was carried out using the Mutan™-Super Express Km kit (Takara Shuzo, Japan) strains were used as host strains to express mutant mGDHs and to introduce the mutation of D354N into the mGDH gene (gcd), respectively. Plasmids used were pUCGCD1 bearing the wild-type gcd (10), pUCGCDK493A bearing K493A gcd, pUCGCDK493R bearing K493R gcd, pUCGCD466E bearing D466E gcd, pUCGCD466N bearing D466N gcd (11), and pUCGCD354N bearing D354N gcd.

Construction of D354N Mutant—To construct the D354N mGDH mutant, site-specific mutagenesis was carried out using the Mutan™-Super Express Km kit (Takara Shuzo) as described previously (11). The mutagenic primer used for the mutant was 5’-CAGTTACCAtaaACTTCTC-3’.

Purification of Wild-type and Mutant mGDHs—UY423 cells harboring the wild-type pUCGCD1 or harboring one of the pUCGCDs encoding mutant mGDH were grown in LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl) containing ampicillin (50 μg/ml) for 12 h at 30 °C under aerobic conditions. Harvesting of cells and preparation of membrane fractions were carried out as described by Yamada et al. (5). Purification of mGDH from membrane fractions was performed at 4 °C as described previously (29) with some modifications. In particular, to improve the recovery of UQ-containing mGDH, we avoided using chloride-containing buffer and Triton X-100, and dialysis after the first column chromatography was eliminated. Membrane fractions (~10 mg/ml of protein) were treated with 10 mM KPB (pH 7.0) containing 0.04% DM and centrifuged 86,000 × g for 90 min, and the resultant membrane fractions were subjected to solubilization for 60 min in the presence of 100 mM KPB (pH 7.0) containing 0.2% DM. The suspension was centrifuged at 86,000 × g for 90 min, and the supernatant obtained was dialyzed against the 10 mM KPB buffer (pH 7.0) without DM. The dialysate was applied onto a DEAE-Toyopearl column (1-ml bed volume/about 5 mg of protein) were treated with 10 mM KPB (pH 7.0) containing 0.04% DM and centrifuged 86,000 × g for 90 min, and the resultant membrane fractions were subjected to solubilization for 60 min in the presence of 100 mM KPB (pH 7.0) containing 0.2% DM. The suspension was centrifuged at 86,000 × g for 90 min, and the supernatant obtained was dialyzed against the 10 mM KPB buffer (pH 7.0) without DM. The dialysate was applied onto a DEAE-Toyopearl column (1-ml bed volume/about 10 mg of protein) equilibrated with 10 mM KPB (pH 7.0) containing 0.1% DM. The column was washed with 10-bed volumes of the same buffer and successively with 10-bed volumes of 10 mM KPB (pH 7.0) containing 0.02% DM. The enzyme was eluted by a linear gradient composed of 10-bed volumes of 25 mM KPB buffer (pH 7.0) containing 0.02% DM and 10-bed volumes of 130 mM KPB (pH 7.0) containing 0.02% DM. Active fractions eluted were pooled and directly applied onto a ceramic hydroxyapatite column (1-ml bed volume/about 5 mg of protein) equilibrated with 10 mM KPB (pH 7.0) containing 0.02% DM. The column was washed with 10-bed volumes of 200 mM KPB (pH 7.0) containing 0.02% DM. mGDH was eluted by a linear gradient composed of 10-bed volumes of 200 mM KPB (pH 7.0) containing 0.02% DM and 10-bed volumes of 1 mM KPB

catalytic reaction and the intramolecular electron transfer in mGDH.

**EXPERIMENTAL PROCEDURES**

**Materials**—UQn was kindly provided by Eizai Co., Ltd. (Japan). All other chemicals were of analytical grade and purchased from commercial sources.

**Bacterial Strains and Plasmids**—E. coli YU423 (Δ(psh psl crr) galP::Tn10 gcd::cm) (8) and MV1184 (Δ(lac-proAB) ara rpsL thi (Δ80 lacZΔM15)Δ(srl-recA) 306:Tn10 (tet’)/F[traD36 proAB lacI1 lacZΔM15]) (Takara Shuzo, Japan) strains were used as host strains to express mutant mGDHs and to introduce the mutation of D354N into the mGDH gene (gcd), respectively. Plasmids used were pUCGCD1 bearing the wild-type gcd (10), pUCGCDK493A bearing K493A gcd, pUCGCDK493R bearing K493R gcd, pUCGCD466E bearing D466E gcd, pUCGCD466N bearing D466N gcd (11), and pUCGCD354N bearing D354N gcd.

**Construction of D354N Mutant**—To construct the D354N mGDH mutant, site-specific mutagenesis was carried out using the Mutan™-Super Express Km kit (Takara Shuzo) as described previously (11). The mutagenic primer used for the mutant was 5’-CAGTTACCAtaaACTTCTC-3’.

**Purification of Wild-type and Mutant mGDHs**—UY423 cells harboring the wild-type pUCGCD1 or harboring one of the pUCGCDs encoding mutant mGDH were grown in LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl) containing ampicillin (50 μg/ml) for 12 h at 30 °C under aerobic conditions. Harvesting of cells and preparation of membrane fractions were carried out as described by Yamada et al. (5). Purification of mGDH from membrane fractions was performed at 4 °C as described previously (29) with some modifications. In particular, to improve the recovery of UQ-containing mGDH, we avoided using chloride-containing buffer and Triton X-100, and dialysis after the first column chromatography was eliminated. Membrane fractions (~10 mg/ml of protein) were treated with 10 mM KPB (pH 7.0) containing 0.04% DM and centrifuged 86,000 × g for 90 min, and the resultant membrane fractions were subjected to solubilization for 60 min in the presence of 100 mM KPB (pH 7.0) containing 0.2% DM. The suspension was centrifuged at 86,000 × g for 90 min, and the supernatant obtained was dialyzed against the 10 mM KPB buffer (pH 7.0) without DM. The dialysate was applied onto a DEAE-Toyopearl column (1-ml bed volume/about 10 mg of protein) equilibrated with 10 mM KPB (pH 7.0) containing 0.1% DM. The column was washed with 10-bed volumes of the same buffer and successively with 10-bed volumes of 10 mM KPB (pH 7.0) containing 0.02% DM. The enzyme was eluted by a linear gradient composed of 10-bed volumes of 25 mM KPB buffer (pH 7.0) containing 0.02% DM and 10-bed volumes of 130 mM KPB (pH 7.0) containing 0.02% DM. Active fractions eluted were pooled and directly applied onto a ceramic hydroxyapatite column (1-ml bed volume/about 5 mg of protein) equilibrated with 10 mM KPB (pH 7.0) containing 0.02% DM. The column was washed with 10-bed volumes of 200 mM KPB (pH 7.0) containing 0.02% DM. mGDH was eluted by a linear gradient composed of 10-bed volumes of 200 mM KPB (pH 7.0) containing 0.02% DM and 10-bed volumes of 1 mM KPB (pH 7.0) containing 0.02% DM and 10-bed volumes of 1 mM KPB.
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(150 mM KPB (pH 7.0) containing 0.1% DM). These concentrated materials were found to have homogeneity of more than 95%, judging from SDS-7% polyacrylamide gel electrophoresis, and were used as purified mGDHs.

Measurement of Protein and Enzyme Activities—Protein content was determined according to the Dulley and Grieve method (35) using bovine serum albumin as a standard. Holoenzyme formation was performed by incubating membrane fractions or purified mGDH in 10 mM MOPS (pH 7.0) containing 30 μM PQQ and 1 mM MgCl₂ for 30 min at 25 °C. Using the holoenzyme thus prepared, the following enzyme activities were measured. PMS reductase activity was measured spectrophotometrically (U-2000A, Hitachi) with PMS and 2,4-dichlorophenol indophenol as an electron mediator and acceptor, respectively, as described previously (3). UQ₂ reductase activity was also measured spectrophotometrically in the presence of 0.0025% Tween 20 (11, 42). The contents of mGDH protein in membrane fractions were compared by Western blot analysis using an antibody against E. coli mGDH (36).

CD Analysis—Far-ultraviolet (200–260 nm) circular CD (far-UV CD) spectra were recorded at 25 °C on a J-600 spectropolarimeter (Jasco) with a 1.0-cm cuvette, by which the conformation of purified mutant mGDHs (2.3 μM) in 10 mM KPB (pH 7.0) was compared with that of the wild-type mGDH.

Determination of UQ Content in Mutant mGDHs—Three nmol of enzyme was treated with 10 volumes of 100% ethanol in the presence of 3 nmol of UQ₆ as an internal standard and incubated for 30 min by mild shaking at 30 °C. The solution was centrifuged at 14,000 rpm for 10 min to remove denatured protein molecules. The supernatant was mixed with 2.5 volumes of n-hexane for 1 min. The upper phase was collected and dried, and the residue was then resolved in 0.2 ml of HPLC solvent (ethanol/methanol/acetonitrile, 4:3:3 v/v). The resolved materials were subjected to reverse-phase HPLC using a Zorbax ODS column (MitsuiToatsu, Japan) at a flow rate of 0.8 ml/min. The elution was monitored at 278 nm by using an SPD-M6A photodiode array detector (Shimadzu, Japan). UQ from purified mGDH samples was identified by comparison of its migration with those of standard UQ₆ (UQ₆ and UQ₉), and the content was estimated from the ratio of the peak area to that of the internal standard UQ₆.

Pulse Radiolysis Analysis of Mutant mGDHs—Pulse radiolysis experiments with purified mutant and wild-type mGDHs were performed on a linear accelerator at the Institute of Scientific and Industrial Research, Osaka University (37–40). The pulse width and energy were 8 ns and 27 MeV, respectively. Samples for pulse radiolysis were prepared in 10 mM potassium phosphate (pH 7.4) and 0.1 M of tert-butyl alcohol (for scavenging OH radicals) and subjected to repeated deaeration followed by flushing with argon gas. Each sample was placed in a quartz cell with an optical path length of 1 cm. The temperature of the sample was maintained at 25 °C. The light source for the spectrophotometer was a 200-watt xenon lamp. After passing through an optical path, the transmitted light intensities were analyzed and monitored by a fast spectrophotometric system composed of a Nikon monochromator, a photomultiplier (Hamamatsu Photonics, R-928), and a Unisoku data analyzing system. For time-resolved transient absorption spectral measurement, the monitor light was focused into quartz optical fiber, which transported the electron pulse-induced transmittance changes to a gated spectrometer (Unisoku, TSP-601-02). The concentration of eaq⁻ generated by pulse radiolysis was determined by absorbance change at 650 nm using an extinction coefficient of 14.1 mM⁻¹ cm⁻¹ (41) and was adjusted by varying the dose of the electron beam.

EPR Spectroscopy—Two hundred μl of concentrated enzyme solution (60–80 μM) in an extra high quality quartz tube (5 mm in outer diameter) was frozen in liquid nitrogen. EPR experiments were carried out on a Bruker E500 spectrophotometer equipped with an hsw10106 resonator and an Oxford E900 cryostat under the same conditions as those described by Elias et al. (29), except that PQQ was not added for detection of the semiquinone radical of bound UQ.

RESULTS

Structural Integrity and Enzyme Activity of Purified Mutant mGDHs—In our previous study, it was shown that Asp-466 and Lys-493, located in close proximity to PQQ, are crucial for a successive process of catalytic reactions from glucose oxidation to UQ reduction. Therefore, we assumed that these amino acid residues interact directly or indirectly with bound UQ, and mutation of these residues appears to impair electron transfer between the bound UQ and PQQ. This assumption was tested with five mutants, D354N, D466E, D466N, K493A, and K493R. Of these, D354N mutant was constructed in this study. Western blot analysis with membrane fractions from cells expressing each mutant mGDH revealed that all mutant mGDHs were expressed at a level similar to that of the wild-type mGDH (11) (data not shown for D354N). In the purification process with two column chromatographies, elution patterns of these mutant mGDHs were found to be almost the same as that of the wild-type mGDH. We further checked effects of mutations on the protein structure by CD analysis with the purified mutant proteins. The results showed that there was almost no difference from that of the wild-type mGDH in the five mutants (Fig. 2 and data not shown), suggesting that these mutants retained a conformation almost unaltered compared with that of the wild-type mGDH.

PMS reductase activity reflecting glucose dehydrogenase activity of purified mGDH mutants was then compared with that of the purified wild-type mGDH (Table 1). As reported previously (10, 11), all mutants except for D354N showed a significantly low level of activity, being less than 0.04% that of the wild-type mGDH, and D354N showed a slightly higher level of activity (about 2.0%) than those of other mutants. Consistent with the level of dehydrogenase activity, UQ₂ reductase activities of these mutants were remarkably reduced. No significant increase both in reductase activities was observed under saturation conditions of electron acceptors in any mutant or wild-type mGDHs. These data suggest that the mutants examined maintain an intrinsic structure of the protein but lose almost all
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Effect of Mutations on UQ Content in Purified mGDHs—If Asp-354, Asp-466, or Lys-493 occurs close to or directly interacts with bound UQ, it is possible that the corresponding mGDH mutants alter the affinity for UQ. To examine this possibility, the content of UQ in each purified mutant mGDH was estimated by reverse-phase HPLC after elution of bound UQ from purified enzymes (Table 1). A compound corresponding to the position of UQ8 was detected at 19 min of retention time, with an absorption spectrum having a peak at 278 nm as reported previously (29). As a result, about 1 mol of UQ8/mol of mGDH protein was detected in the case of K493R mutant, but about 0.5, 0.2, 0.1, and 0.1 mol of UQ8/mol of mGDH protein were found in D354N, D466N, D466E, and K493A, respectively. These observations suggest that amino acid substitutions of Asp-354, Asp-466, and Lys-493 (K493A) alter the affinity for UQ at the Q1 site.

Pulse Radiolysis Analysis of Mutant mGDHs—Pulse radiolysis is a powerful tool for investigating electron transfer within a protein, often allowing an electron to be introduced rapidly and selectively into one redox center of an enzyme. In our previous study using wild-type mGDH, a radiolytically generated eaq reacted predominantly with bound UQ to form a UQ semiquinone neutral radical (UQH+), and intramolecular electron transfer from UQ semiquinone to PQQ subsequently occurred (31). The assignments were confirmed by the evidence that such changes were not observed in the UQ-free enzyme or in PQQ-free apoenzyme (31). We thus examined, using purified holoenzymes, the effect of amino acid substitutions of mGDH mutants on the intramolecular electron transfer from bound UQ to PQQ and the formation of a semiquinone radical of bound UQ. As in the case of the wild enzyme, the bound UQ of these mutants was initially reduced. However, spectral properties of the UQ semiquinone and the decay process were quite different for the mutants. As in the case of the wild-type enzyme, for the K493R mutant, a UQ semiquinone neutral radical with an absorption maximum at 420 nm was observed (Fig. 3, B and C, insets), and subsequently, a decay in the absorption at 420 nm was accompanied by absorption increase around 370 nm. This indicates that intramolecular electron transfer from the UQ to PQQ occurred. The UQ semiquinone neutral radical, however, was very stable compared with that of the wild-type mGDH (Fig. 3, A and B, insets). The rate constant for the decay of the semiquinone in the K493R mutant (8.5 × 102 s−1) was found to be slower than that in the wild-type enzyme (3.2 × 103 s−1). On the other hand, it is noteworthy that the transient spectra initially formed in the D354N and D466N mutants differ in maxima. The spectral properties of the intermediate are similar to those of UQ semiquinone anion radical. Similarly, our pulse radiolysis experiment for cyto-

![CD spectra of D466N, D493R, and wild-type mGDHs. Far-ultraviolet CD spectra of wild type (A), D466N (B), and K493R (C). All samples contained 10 mM KPB (pH 7.0) and 2.3 μM apoenzyme.](image)

![Table 1.](image)
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Transient absorption spectra taken at 50 μs (red) and 500 μs (blue) after pulse radiolysis of wild-type (A), K493R (B), D354N (C), and D466N (D) mGDHs. All samples contained 10 mM KPB (pH 7.4), 0.1 mM tert-butyl alcohol, and 48 μM holoenzyme. Inset, absorption changes after pulse radiolysis of holoenzymes.

FIGURE 3. Kinetic difference of absorption spectra of D354N, D466N, K493R, and wild-type mGDHs. The results presented here provide the following important implication for the catalytic mechanism of the enzyme. The D466N mutant formed a UQ semiquinone anion radical, whereas the semiquinone radical in the wild-type mGDH was predicted to be involved in the intramolecular electron transfer process. The results presented here provide the following important implication for the catalytic mechanism of the enzyme. The D466N mutant formed a UQ semiquinone anion radical, whereas the semiquinone radical in the wild-type mGDH was predicted to be involved in the intramolecular electron transfer process.

Among membrane-bound quinoprotein dehydrogenases, mGDH is the first enzyme that has been shown to possess a tightly bound UQ in the molecule (29). This study focused on amino acid residues that reside close to the orthoquinone portion of PQQ, which are assumed to interact with the bound UQ on the basis of the finding of a very small distance between bound UQ and PQQ (31). Among such residues, it has been proposed that Asp-466 plays a crucial function in extraction of a proton from glucose during the catalytic reaction; Lys-493 hydrogen-bonds to orthoquinone of PQQ, and Asp-354 interacts with a metal ion in the reaction center (14). Although bound UQ in the mGDH has speculated to be involved in the intramolecular electron transfer (29), its interaction with surrounding amino acid residues has not been reported. The amino acid-substituted mutants, D466N, D466E, and K493A, caused a large reduction in content of bound UQ. The results shown in Table 1 imply that for the adaptation of UQ, the positive charge on Lys-493 and the negative charge on Asp-466 with appropriate distance are critical, and the negative charge on Asp-354 also makes some contribution. Pulse radiolysis analysis revealed that K493R stabilized a semiquinone neutral radical of bound UQ and that D354N and D466N exhibited semiquinone anion radicals but not neutral radicals. D466N and D466E showed an undefined EPR signal in addition to the signals observed in the wild-type mGDH. Moreover, all of these mutations remarkably reduced activities of dehydrogenase and UQ₂ reductase, and D466N, K493R, and D354N mutations prevented the formation of PQQH₂ following the addition of a substrate (Ref. 11 and data not shown). These findings suggest that these three amino acid residues have interaction with bound UQ in addition to PQQ and are involved in the catalytic reaction and/or the following event in the intramolecular electron transfer process.

The results presented here provide the following important implication for the catalytic mechanism of the enzyme. The D466N mutant formed a UQ semiquinone anion radical, whereas the semiquinone radical in the wild-type mGDH was assigned as a neutral form. This finding allows us to simply speculate that Asp-466 removes a proton from glucose and

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passes it to the bound UQ that has received an electron from the reduced PQQ. If this is the case, the proton transfer from Asp-466 to bound UQ may be a crucial step for the next round of substrate oxidations. Although the D354N mutant also formed a UQ semiquinone anion radical, Asp-354 would indirectly interact with bound UQ. Asp-354 is located far from the ortho-quinone portion of PQQ and interacts with a metal ion. The mutation may alter the microenvironment around Asp-466 by change in the interaction with the metal ion, which in turn hampers the proton donation to UQ.

Interestingly, on the other hand, K493R was found to significantly stabilize a UQ semiquinone neutral radical. The rate constant for the backward interdomain electron transfer was affected. Previously, we have proposed that Lys-493, hydrogen bonding with the C-4 and C-5 carbonyl oxygen of PQQ, has an important role of electron transfer from PQQH₂ to the bound UQ (11). This is consistent with our proposal. Alternatively, the mutation may influence the microenvironment around Asp-466 by change in the interaction with the metal ion, which in turn hampers the proton donation to UQ.

The UQ semiquinone radical in PQQ-free apo-forms of mGDH is first observed by EPR spectroscopy. The sharp signal with $g = 2.004$ and about 10 G of peak-to-peak line width is characteristic of UQ radicals, similar to those observed in the bo$_{3}$-ubiquinol oxidase ($g_{\text{iso}} = 2.0044, \sim 10$ G width (43)) or in the E29L mutant of quinol-fumarate reductase ($g = 2.005, \sim 11$ G width (44)). The newly detected broad singlet signal(s) in mGDH, which will be characterized in future works, indicates that most of the paramagnetic UQ molecules are in a similar situation both in the wild-type and D354N mutant molecules. Because the UQ content both of the wild type and K493R is $\sim 100\%$, the content of paramagnetic UQ in K493R is estimated to be lower than that of the wild type, i.e. mutation on Lys-493 has largely modified the oxidation state of bound UQ. Similarly, the D466N mutant, which has only 20% of bound UQ, exhibits a relatively high content of paramagnetic quinone species in the EPR spectrum, whereas the D466E mutant, which has $\sim 10\%$ of bound quinone content, has much less paramagnetic UQ.
the oxidation state of bound UQ is also altered by this mutation. Furthermore, mutation on the Asp-466 residue gave rise to an additional sharp signal in the EPR spectrum, which means that another state of bound UQ radical was created by the mutation on Asp-466. Both D466N and D354N similarly showed semiquinone anion radicals in pulse radiolysis but exhibited distinctive EPR spectra. Considering closer location of Asp-466 to bound UQ as discussed above, the additional sharp EPR signal in D466N would be due to alteration of the position of bound UQ by mutation in some mGDH molecules. The observed changes in EPR spectra brought about by these mutations strongly suggest that the Lys-493 and Asp-466 residues are in close proximity of bound UQ and work for not only keeping UQ in mGDH molecules but also mediating electron transfer between UQ and PQQ.

Purified samples may contain three different states of mGDH molecules bearing semiquinone and reduced and oxidized bound UQ. The first two states have been indicated by EPR before and after, respectively, the addition of ferricyanide in the bulk-reduced quinone to become semiquinone species, and it is likely that some bound UQ molecules accept electrons from PQQ, a primary electron donor for the bound UQ in mGDH, it may be one reason why E. coli lacking PQQ-synthesizing genes retains the mGDH gene.

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