# Mutagenesis of Three Conserved Glu Residues in a Bacterial Homologue of the ND1 Subunit of Complex I Affects Ubiquinone Reduction Kinetics but Not Inhibition by Dicyclohexylcarbodiimide†

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ABSTRACT: Steady-state kinetics of the $H^+$-translocating NADH:ubiquinone reductase (complex I) were analyzed in membrane samples from bovine mitochondria and the soil bacterium *Paracoccus denitrificans*. In both enzymes the calculated $K_m$ values, in the membrane lipid phase, for four different ubiquinone analogues were in the millimolar range. Both the structure and size of the hydrophobic side chain of the acceptor affected its affinity for complex I. The ND1 subunit of bovine complex I is a mitochondrially encoded protein that binds the inhibitor dicyclohexylcarbodiimide (DCCD) covalently [Yagi and Hatefi (1988) *J. Biol. Chem. 263*, 16150−16155]. The NQO8 subunit of *P. denitrificans* complex I is a homologue of ND1, and within it three conserved Glu residues that could bind DCCD, E158, E212, and E247, were changed to either Asp or Gln and in the case of E212 also to Val. The DCCD sensitivity of the resulting mutants was, however, unaffected by the mutations. On the other hand, the ubiquinone reductase activity of the mutants was altered, and the mutations changed the interactions of complex I with short-chain ubiquinones. The implications of the results for the location of the ubiquinone reduction site in this enzyme are discussed.

The $H^+$-translocating NADH:ubiquinone oxidoreductase (complex I) is a large membrane-bound enzyme that catalyzes the first electron-transfer reaction of the respiratory chain in most mitochondria and several bacteria. The mitochondrial enzyme is located in the inner membrane and contains some 42 protein subunits, 7 of which are encoded and synthesized within the organelle (*1*). The latter proteins, called ND1−ND6 and ND4L, form most of the membrane domain of the enzyme and thus are probably involved in ubiquinone binding and proton translocation. Mutations in several mitochondrially encoded subunits (the ND's) may cause a number of neurodegenerative disorders and might be involved in aging (*2*, *3*). It is thus interesting to study the effect of mutations in different ND's on the enzymatic activity of complex I. Such an undertaking is hampered, however, by the enormous difficulties to introduce specific mutations into mitochondrially encoded proteins.

Bacterial complexes I contain 14 different protein subunits, 7 of which are homologues of the mitochondrially encoded subunits of the eucaryotic enzyme (*4*). In the case of the soil bacterium *Paracoccus denitrificans* there is high homology in the primary structure of all its complex I subunits and their mitochondrial counterparts (*5*). In addition, this bacterial enzyme closely resembles bovine complex I with respect to the properties of most of its Fe−S clusters (*6*−*8*). Nevertheless, we have recently demonstrated that the ferricyanide reductase activity of *P. denitrificans* complex I differs from that of its mitochondrial counterpart (*9*). Both enzymes, however, reduce hexaammineruthenium (HAR)[1] similarly (*9*).

Bacterial complexes I provide attractive systems for studies on the role of specific residues in the central subunits of the enzyme using mutagenesis, and we have previously used *P. denitrificans* for a similar purpose (*10*). Important information about the structure and function of complex I was also obtained from genetic manipulation of the filamentous fungus *Neurospora crassa* (*11*; see ref *12* for a recent review), but the bacterial enzymes have a clear advantage when the target protein is mitochondrially encoded in eucaryotes. Understanding the effects and implications of engineered mutations in the ND subunits of the enzyme for its structure and function requires good knowledge about the kinetics of ubiquinone reduction in both bacterial and mitochondrial complex I. We have undertaken such a study and analyzed the steady-state kinetics of the rotenone-sensitive reduction of short-chain ubiquinone analogues in membranes from *P.*

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[1] Abbreviations: DB, decylubiquinone (formerly decylbenzoquinone); DCCD, dicyclohexylcarbodiimide; HAR, hexaammineruthenium; $Q_1$, ubiquinone-1; $Q_2$, ubiquinone-2; $Q_3$, ubiquinone-3.

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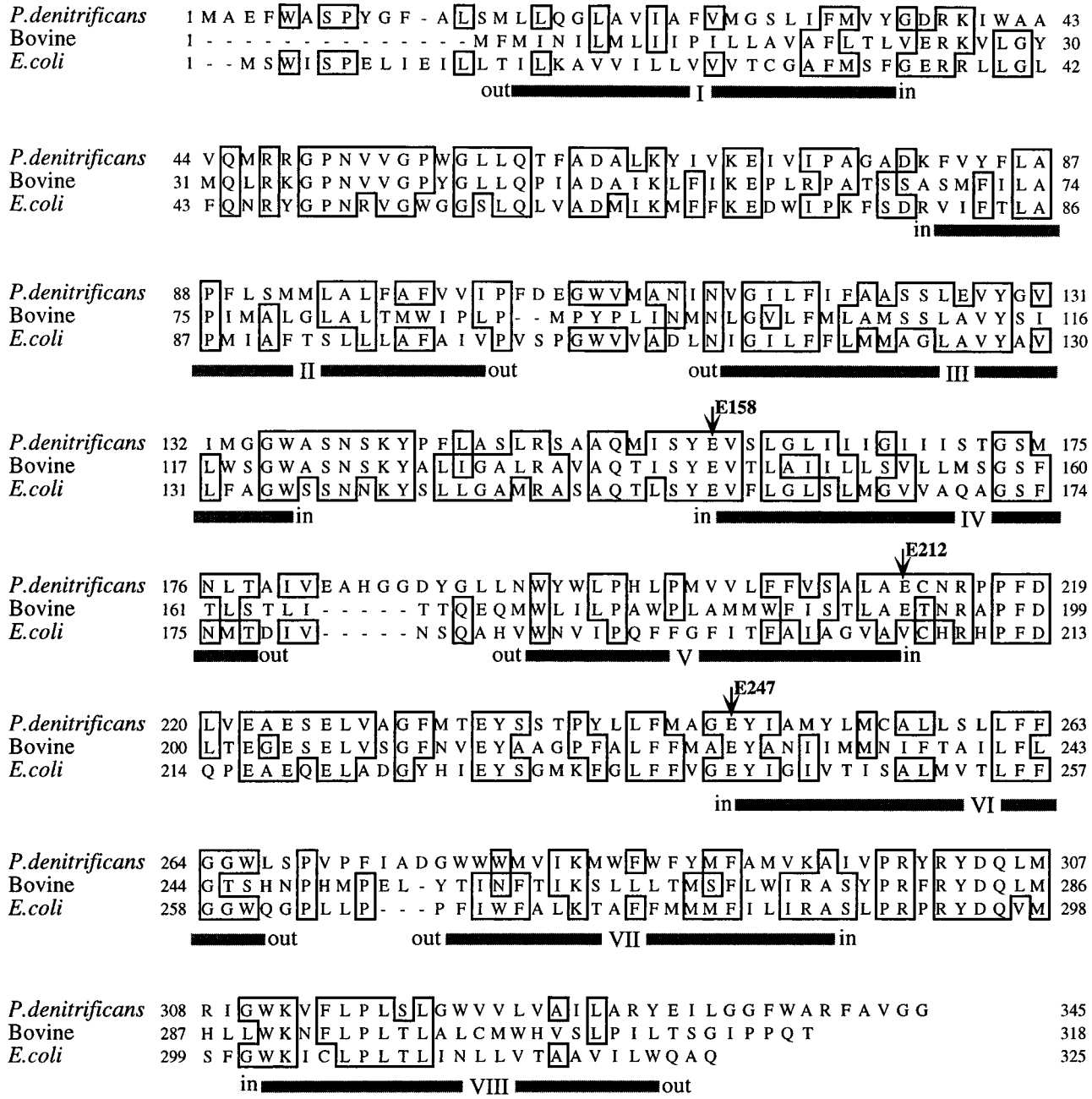


FIGURE 1: Alignment of the NQO8 subunit of *P. denitrificans* complex I with the ND1 subunit of bovine complex I and the NUOH subunit of the enzyme from *E. coli*. The thick gray lines under the sequences indicate positions of putative transmembrane helices and their suggested orientation with respect to the cytoplasm of the bacterial cell (matrix side in mitochondria). The three Glu residues that were mutated in this study are indicated by arrows. Genebank accession numbers for the presented sequences are P29920 (*P. denitrificans*), P03887 (bovine), and P33603 (*E. coli*).

*denitrificans* and bovine heart mitochondria, the results of which are presented and discussed below.

Dicyclohexylcarbodiimide (DCCD) inhibits the quinone reductase activity of complex I from different organisms (*13*, *14*). In *P. denitrificans* complex I DCCD also inhibits ferricyanide reduction, although to a lower degree than ubiquinone reduction (*9*, *14*). DCCD may bind covalently to the protein, and in the case of bovine complex I it was reported that inhibition correlated with DCCD binding to the ND1 subunit (*15*). In another study, however, DCCD bound to the "ASHI" subunit, a nuclear-encoded protein that has no homologue in bacterial complex I (*16*). In several other enzymes, e.g., the $F_1$-$F_0$ ATP synthase and cytochrome *c* oxidase, DCCD binds to a highly conserved acidic residue, mostly Glu, that is located in the middle of a (predicted) transmembrane α-helix (see ref 16 for a review). A recent addition to the list of DCCD-sensitive enzymes is EmrE, a proton−multidrug antiporter from *Escherichia coli*, in which a glutamyl residue in the middle of a transmembrane helix binds the inhibitor (*17*). Hence, it was interesting to try to locate the DCCD-binding amino acid in complex I since that residue may play an important role in the proton translocating activity of the enzyme. Subunit NQO8 (or NUOH) of bacterial complex I is a homologue of the mitochondrial ND1 subunit, and we have looked for conserved acidic residues within the 8 putative transmembrane helices of the *P. denitrificans* protein (Figure 1). No such amino acid was identified in NQO8, but it appeared that the E158, E212,

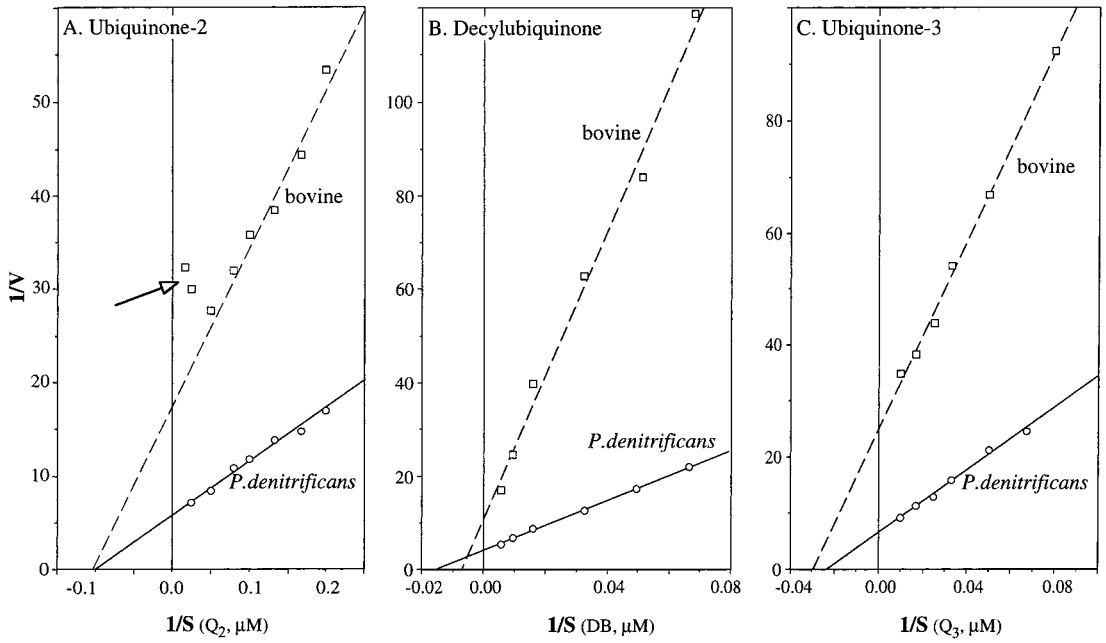


FIGURE 2: Double reciprocal plots of ubiquinone reductase activities (rotenone sensitive) of complex I in membranes from *P. denitrificans* and bovine heart mitochondria. The rates of NADH oxidation in the presence of either $Q_2$ (A), DB (B), or $Q_3$ (C) are presented relative to the respective rates of HAR reduction in the same membrane preparations. The arrow in (A) highlights the inhibition of bovine complex I by high concentrations of $Q_2$ (substrate inhibition).

and E247 residues of this subunit are situated within relatively hydrophobic stretches of the protein (Figure 1) and are thus potential targets for DCCD. In addition, they are probably located on the inner surface of the membrane (Figure 1), i.e., in the vicinity of a region that is involved in ubiquinone binding (*10*). To examine the involvement of these Glu residues in DCCD inhibition and ubiquinone reduction, we have replaced them by either Asp or Gln (and also Val in the case of E212) and analyzed the inhibitor sensitivity and steady-state kinetics of the resulting complex I mutants, as described below.

## MATERIALS AND METHODS

*Materials*. Ubiquinone-1 ($Q_1$) was a generous gift from Hofmann-La Roche, Basel, Switzerland; ubiquinone-2 ($Q_2$) and ubiquinone-3 ($Q_3$) were generous gifts from Eisai, Tokyo, Japan; decylubiquinone (DB), antimycin A, and deamino-NADH were purchased from Sigma, rotenone was from Aldrich, and NADH was from Boehringer.

Site-specific mutagenesis was essentially carried out according to the procedure of Vandeyar et al. (*18*), and the transfer of the mutated *nqo8* gene into the deletion strain, Pd92-223 (*19*), was carried out as previously described (*10*). The presence of correct mutations in the genomic DNA of the isolated mutant strains was verified by DNA sequencing of amplified *nqo8* or by restriction analyses in case the respective mutation introduced a suitable site.

*Preparative Procedures*. The *P. denitrificans* strains F2 (*10*) and MK6 (*20*), and the mutants, were grown in a malate-containing medium, and membranes were prepared from mid-log-phase cells as described previously (*10*). The presence or absence of an active NDH-2 in the membrane preparations was determined by following NADH and deamino-NADH oxidation, as previously described (*17*). Membranes from bovine heart mitochondria were prepared according to ref *21*.

*Activity Assays*. Enzymatic activities were assayed at 30 °C using a Shimadzu UV3000 or a Cary 1C spectrophotometer. HAR reductase activity was measured as described previously (*10*). The reaction mixture for assays of $Q_1$, $Q_2$, $Q_3$, and DB reductase activity contained 1 mg/mL sonicated soybean phospholipid, 50 mM $KP_i$, pH 7.0, 0.2 mM NADH or deamino-NADH, and different amounts of acceptor. In addition, it contained 5 mM KCN (neutralized with HCl) and antimycin A at either 1.25 $\mu$M (mitochondrial membranes) or 4.5 $\mu$M (*P. denitrificans* membranes). It may be noted that antimycin A was not present in our previous study (*10*).

Assays of $Q_1$ and $Q_2$ reduction were started by the addition of the acceptor to the reaction mixture that contained NADH (or deamino-NADH), while in the case of DB and $Q_3$ the electron donor was added after 3−5 min incubation in the presence of the acceptor. The activity measurements in the presence of inhibitors were started after preincubation for 5 min at 30 °C before the addition of either $Q_1$, $Q_2$, or NADH (when the acceptor was DB or $Q_3$). All of the ubiquinone reductase measurements were stopped by the addition of rotenone to a final concentration of 30 $\mu$M, and the rotenone-insensitive rate of NADH oxidation was subtracted. Inhibition of the $Q_1$ reductase activity by DCCD was assayed as described previously (*9*).

Protein concentrations were determined according to the method of Lowry et al. (*22*) in the presence of 0.2% SDS.

## RESULTS

The ubiquinone reductase activity of bacterial and mitochondrial complex I was studied using enzymes in the native membranes from *P. denitrificans* and bovine heart mitochondria. We have analyzed the steady-state kinetics of rotenone-sensitive reduction of $Q_1$, $Q_2$, $Q_3$, and DB, and the results are presented in Figure 2 and Table 1. The HAR reductase activity of complex I was used as a measure for

Table 1: $K_m$ and $V_{max}$ Values for the Reduction of Short-Chain Ubiquinones by Complex I from Bovine Heart Mitochondria and *P. denitrificans*[a]

| acceptor | kinetic parameters | bovine | *P. denitrificans* |
|---|---|---|---|
| $Q_1$ | $K_{m(app)}$ ($\mu$M) | 48 | 16.5 |
| | $K_{m(calc)}$[b] (mM) | 21.3 | 7.3 |
| | $V_{max}$ ($Q_1$/HAR) | 0.21 | 0.23 |
| $Q_2$ | $K_{m(app)}$ ($\mu$M) | 9.5 | 10.0 |
| | $K_{m(calc)}$[b] (mM) | 8.6 | 9.1 |
| | $V_{max}$ ($Q_2$/HAR) | 0.06 | 0.19 |
| DB | $K_{m(app)}$ ($\mu$M) | 157.1 | 69 |
| | $K_{m(calc)}$[b] (mM) | 154 | 68 |
| | $V_{max}$ (DB/HAR) | 0.1 | 0.26 |
| $Q_3$ | $K_{m(app)}$ ($\mu$M) | 34 | 42.9 |
| | $K_{m(calc)}$[b] (mM) | 33.5 | 42.2 |
| | $V_{max}$ ($Q_3$/HAR) | 0.037 | 0.15 |

[a] The values were derived from the data shown in Figure 2. [b] The $K_{m(calc)}$ values were calculated from $K_{m(app)}$ using the partition coefficients determined by Fato et al. (*23*, *24*). The partition coefficients, $P$ [(moles of Q per liter of phospholipid)/(moles of Q per liter of water)], were as follows: $Q_1$, log $P = 2.9$; $Q_2$, log $P = 4.0$; $Q_3$, log $P = 4.8$; DB, log $P = 4.7$. The formula used for calculation (*23*) was $[S]_L = P[S]_{added}/P\alpha + 1 - \alpha$, where $[S]_L$ is the substrate concentration in the lipid phase, $P$ is the partition coefficient, and $\alpha$ is the fractional volume, $V_{lipid}/V_{total}$.

the enzyme concentration in each membrane sample (*9*, *10*), and the enzyme activities for the plots (Figure 2) were obtained by dividing the rate of NADH (or deamino-NADH) oxidation in the presence of the ubiquinone analogues by the rate of HAR reduction of the same sample of washed membrane. The measurements were done in the presence of antimycin A and KCN in order to keep internal ubiquinone-10 pools in the fully reduced state throughout the assays and to minimize electron transfer from the cytochrome $bc_1$ complex to the added short-chain quinones.

The concentration of $Q_1$, $Q_2$, $Q_3$, and DB in the membrane was determined by the partition coefficient of each acceptor between the bilayer and the aqueous phases and the fractional volumes of phospholipid in the cuvette (*23*). We have measured the reduction rate of these acceptors in the presence of sonicated soybean phospholipid that was added to the extent that the contribution of phospholipid in the membrane sample was practically negligible. This allowed calculation of the acceptor concentration in the lipid phase using known partition coefficients (*23*, *24*) and the phospholipid concentration in the cuvette (Table 1). The present results regarding the $K_m$ of bovine complex I for short-chain ubiquinones (Table 1) are in good agreement with a previous study that used a different method to calculate the fractional volume of the phospholipid bilayer in the sample (*23*). This highlights the suitability of the current method for kinetic analyses of complex I in samples, the phospholipid/protein content of which is variable, such as bacterial membranes from different preparations.

The quinone reductase activity of mitochondrial and bacterial complex I is sensitive to DCCD (*9*, *13*−*16*). In bovine complex I the inhibition by DCCD correlated with binding of labeled inhibitor to the ND1 subunit (*14*, *15*). However, the specific amino acid that binds DCCD in this subunit has not been identified. We have used site-specific mutagenesis in order to find out whether three conserved Glu residues in NQO8, the *P. denitrificans* homologue of ND1 (Figure 1), are involved in DCCD binding. Each of these residues was changed to either Asp or Gln, and E212 was also changed to Val since this is the amino acid found at this position in the *E. coli* counterpart of ND1, i.e., V206 of nuoH (Figure 1). NuoH of *Salmonella typhimurium* (*25*) and *Klebsiella pneumoniae* (can be found using the search facilities at http://genome.wustl.edu/gsc/Projects/bacterial/klebsiella/klebsiella.shtml) also have Val in this position, but except for these almost identical proteins no other homologue of ND1 in the database was found in which the respective amino acid was not Glu (not shown).

Table 2: Characterization of the *P. denitrificans* NQO8 Mutants[a]

| mutant | mutant colonies without an active NDH-2 | HAR reductase activity | $Q_1$ reductase activity | $Q_1$/HAR ratio of activities |
|---|---|---|---|---|
| E158D | 0/6 | 2.246 ± 0.310 | 0.216 ± 0.004 | 0.096 |
| E158Q | 0/5 | 1.821 ± 0.073 | 0.142 ± 0.003 | 0.078 |
| E212D | 3/3 | 1.901 ± 0.330 | 0.177 ± 0.013 | 0.093 |
| E212Q | 1/3 | 5.030 ± 0.096 | 0.312 ± 0.032 | 0.062 |
| E212V | 1/4 | 2.390 ± 0.086 | 0.273 ± 0.069 | 0.114 |
| E247D | 3/4 | 1.556 ± 0.240 | 0.145 ± 0.007 | 0.093 |
| E247Q | 0/6 | 1.363 ± 0.176 | 0.119 ± 0.002 | 0.087 |
| F2 (control) | | 1.144 ± 0.060 | 0.161 ± 0.013 | 0.141 |

[a] The presence of an active NDH-2 was assayed polarographically. The values indicate the number of colonies of the given mutant that exhibited no NDH-2 activity out of the total number of mutant colonies found in each case. Complex I activities were measured under standard conditions (*9*, *10*) using deamino-NADH as the electron donor. The values are the means of at least three measurements ± standard deviation. The activity units are $\mu$mol of deamino-NADH oxidized·(mg of protein)$^{-1}$·min$^{-1}$.

The mutated genes were transferred to the *P. denitrificans* strain that lacks the *nqo8* and *nqo9* genes and contains, in their place, the *ndh* gene that encodes NDH-2 of *E. coli* (*19*). The correct incorporation of the mutated genes into the operon of Pd92-223 was examined by the color of the colony and its resistance to certain antibiotics (*10*). The presence or absence of an active NDH-2 was determined in membranes that were prepared from the mutant colonies (*19*), and the results are listed in Table 2. In cases where a colony harbored the mutated *nqo8* gene and no active NDH-2, i.e., mutants E212D, E212Q, E212V, and E247D, one of the NDH-1-only colonies was selected for further analyses. Otherwise, for mutants E158D, E158Q, and E247Q, further analyses were done in the presence of active NDH-2, and hence it was essential to use deamino-NADH as the electron donor in these cases. In addition, we have recently reported that the rate of the HAR reductase activity of *P. denitrificans* is higher with deamino-NADH than with NADH (*9*), and due to this deamino-NADH was used in the subsequent analyses of all the mutants (Tables 2 and 3).

The level of complex I expression in each mutant was assessed from its specific HAR reductase activity, and the $Q_1$/HAR ratio of activities provided an indication about the severity of the mutation for ubiquinone reduction (Table 2 and ref *10*). It should be pointed out here that in the current study the apparent $Q_1$ reductase activity of the control strain, F2, and hence its ratio of activities, is lower than in our previous work (*10*). These differences are due to the addition of antimycin A to the assay mixture in the present study, which seems to block $Q_1$ reduction by the fully reduced cytochrome $bc_1$ complex. Addition of antimycin A to the assay lowered the $V_{max}$ value of *P. denitrificans* complex I in the presence of $Q_1$ and $Q_2$ by about 30% but did not affect

Table 3: DCCD Inhibition of the $Q_1$ Reductase Activity of the Control (F2) *P. denitrificans* Complex I and Mutants E247D and E247Q[a]

| | mutant | DCCD inhibition (%) |
|---|---|---|
| pH 7.0 | E247D | 81 |
| | E247Q | 71 |
| | F2 (control) | 76 |
| pH 8.0 | E247D | 95 |
| | E247Q | 84 |
| | F2 (control) | 91 |

[a] The inhibition of the deamino-NADH:$Q_1$ reductase activity was calculated in comparison to the activity in parallel samples that were incubated similarly, except that DCCD was replaced by the appropriate amount of ethanol.

its $K_m$. Direct inhibition of the bacterial complex I by antimycin A was, however, excluded since the addition of this complex III inhibitor had no effect on complex I activity in membranes from MK6, a *P. denitrificans* strain that lacks the cytochrome $bc_1$ complex (*20*) (results not shown).

Examinations of complex I and NDH-2 activities in the mutants indicated that E212Q has an exceptionally high expression level (Table 2). This may explain the presence of one E212Q colony without an active NDH-2, despite the low ratio of activities ($Q_1$/HAR) in this mutant (Table 2). Among the two E158 mutants there was not a single colony without an active NDH-2, even though both the ratio of activities and the expression level in E158D were at least as high as in E212D where all the mutant colonies grew without NDH-2 (Table 2). The reason(s) for these findings is(are) currently unclear. The results of mutations at E247 suggest that either the glutamic or aspartic acid residue is needed at this position to allow bacterial growth without NDH-2 and that glutamine cannot support such a growth (Table 2).

The reliance of complex I-dependent growth on the presence of an acidic residue at position 247 of *nqo8* (Table 2) made the E247D and E247Q mutants particularly interesting subjects for DCCD inhibition experiments. This was examined after 16 h at 0 °C in either the presence or absence of 800 $\mu$M DCCD at two different pH values, 7.0 and 8.0. The results (Table 3) show that, within the experimental error of the experiment, the sensitivity of the $Q_1$ reductase activity of the two mutants to DCCD is similar to the control. It may be added that the partial inhibition, 30−40%, of the ferricynide reductase activity of both mutant enzymes was practically the same as in wild-type bacterial complex I (*9*). Similar results were also obtained for all of the other mutants, and it can thus be concluded that none of the Glu residues examined here, i.e., E158, E212, and E247 of the NQO8 subunit of *P. denitrificans* complex I, is directly involved in DCCD inhibition.

The low ratio of $Q_1$/HAR reduction rates of the mutants (Table 2) may indicate that the three Glu residues that were mutated in this study are involved in ubiquinone reduction. Hence, we have analyzed the steady-state kinetics of the $Q_1$ and $Q_2$ reductase activities (rotenone sensitive) of all the mutants, and the results are summarized in Table 4. In addition, the double reciprocal plots of the $Q_2$ titrations are presented in Figure 3 in order to demonstrate that none of the new mutants was inhibited by high concentrations of this acceptor (substrate inhibition).

GenBank searches reveal that E158 is strictly conserved in ND1 of complex I. E247 is fully conserved in animal and bacterial complex I, but in the ND1 homologue encoded by chloroplast DNA and in cyanobacteria, it is replaced by Ser (not shown). E212 is conserved in most species, including plants, but in *E. coli* NUOH it is replaced by Val (Figure 1). Interestingly, complex I from *E. coli* is much less sensitive to rotenone and piericidin A than several other bacterial complexes I (*8*), and it was previously suggested that rotenone binds to the ND1 of the enzyme (*26*). We have thus examined the rotenone sensitivity of mutant E212V in order to find out whether this residue is involved in rotenone inhibition. The $Q_1$ reductase activity of the mutant and the control enzyme was measured in the presence of different concentrations of rotenone, followed by a second addition of rotenone (to 30 $\mu$M) that should fully inhibit the enzyme and allow subtraction of the rotenone-insensitive activity. It should be

Table 4: Kinetic Parameters for the Quinone Reductase Activity of the E158, E212, and E247 Mutants (*P. denitrificans* Complex I, NQO8 Subunit)[a]

| | $Q_1$ | | $Q_2$ | |
|---|---|---|---|---|
| mutant | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| E158D | 19.0 | 0.125 | 5.6 | 0.090 |
| E158Q | 23.9 | 0.100 | 12.0 | 0.067 |
| E212D | 16.8 | 0.120 | 12.9 | 0.125 |
| E212Q | 27.7 | 0.090 | 20.0 | 0.110 |
| E212V | 9.6 | 0.125 | 4.6 | 0.139 |
| E247D | 19.7 | 0.110 | 25.1 | 0.083 |
| E247Q | 5.7 | 0.080 | 7.3 | 0.050 |
| F2 (control) | 16.5 | 0.192 | 12.5 | 0.167 |

[a] The $K_m$(apparent) (in $\mu$M) and the $V_{max}$ (relative to HAR reduction rate) values were derived from double reciprocal analyses of acceptor titrations, e.g., Figure 3.

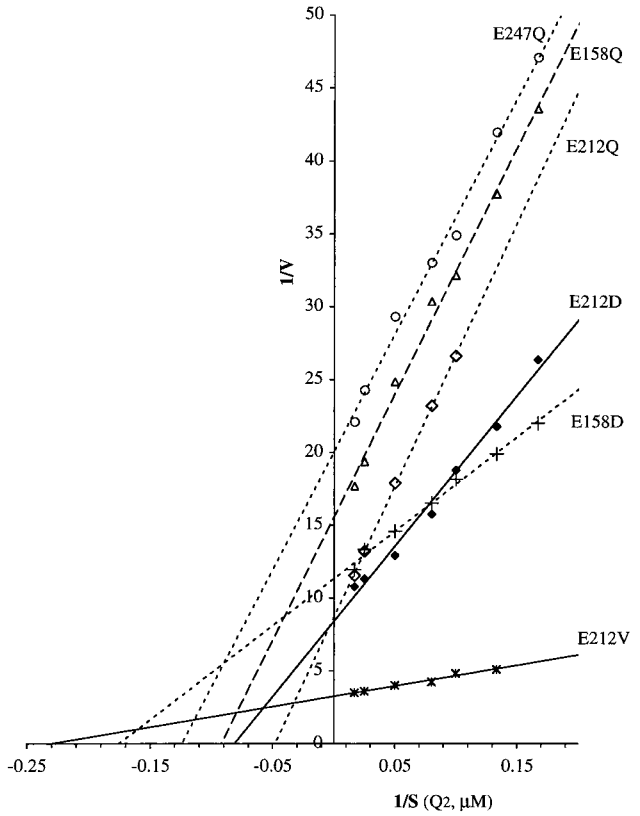


FIGURE 3: Double reciprocal plot of the $Q_2$ reductase activity (rotenone sensitive) of *P. denitrificans* complex I mutants. The specific mutations in the NQO8 subunit of the enzyme are indicated for each extrapolation line. The electron donor was deamino-NADH.

pointed out that the assay mixture contained sonicated phospholipid, as in all our ubiquinone reductase activity measurements reported here, and the acceptor concentration was 60 mM $Q_1$. The inhibition curves for both the E212V mutant and the control (F2) membranes were very similar, and the inhibitor concentration that resulted in 50% inhibition ($I_{50}$) under the assay conditions was about 2 $\mu$M rotenone.

## DISCUSSION

In this work we have addressed the question of how do the large differences in size and subunit composition, as well as variations in primary structure of the subunits, between bovine mitochondrial complex I and the enzyme from *P. denitrificans* affect their interactions with ubiquinone. The results (Figure 2 and Table 1) suggest that, besides the similarities, there are interesting differences between the two enzymes. The structure of the quinone side chain affects its affinity for both enzymes but not to the same degree. The $K_m$ values of bovine complex I, as well as its $V_{max}$, are influenced by the side chain of the acceptor much more than the respective values in the bacterial enzyme (Table 1). The affinity of complex I from both mitochondria and bacteria for ubiquinone analogues is rather low, $K_m$ values in the millimolar range, assuming that the acceptor concentration in the membrane bilayer is the relevant one (Table 1). This low affinity may reflect the importance of the pool property of ubiquinone for optimal function of the respiratory chain as a whole, rather than of a single enzyme.

The DB reductase activity of *P. denitrificans* complex I was previously found to be very low (*27*). The present results, however, are at variance with this, and the $V_{max}$ in the presence of DB was actually the highest found for *P. denitrificans* complex I (Table 1). Interestingly, the $K_m$ of the DB reductase activity (in both mitochondria and bacteria) is significantly higher than for $Q_1$ and $Q_2$ (Table 1), suggesting that the quinone binding site of complex I distinguishes between aliphatic and isoprenoid side chains. Nevertheless, these high $K_m$ values cannot explain the poor DB reductase activity that was previously reported for *P. denitrificans*, but not bovine, complex I (*27*).

Complex I from bovine (and human) mitochondria is inhibited by high concentrations of $Q_2$ (*23*, *27*, *28*; also pointed out by the arrow in Figure 2A). Bacterial complex I from *P. denitrificans* does not exhibit such substrate inhibition (Figure 2A), unless it carries some specific mutations (*10*). The present work demonstrates that neither DB nor $Q_3$ elicits substrate inhibition on either bovine or *P. denitrificans* complex I (Figure 2).

The inhibition of complex I from bovine heart mitochondria and several bacteria by DCCD has been studied previously (*9*, *13*−*16*). The report that DCCD binds to the ND1 subunit of the mitochondrial enzyme prompted Hassinen and Vuokila to suggest that E143 of the bovine protein (i.e., E158 in *P. denitrificans*, Figure 1) may be involved in DCCD binding (*16*). In the present work we have used site-specific mutagenesis to replace this strictly conserved amino acid, as well as E212 and E247 (*P. denitrificans* sequence), by either Asp or Gln. None of the mutants were resistant to DCCD inhibition (Table 3 and unpublished results), indicating that these acidic residues do not bind DCCD.

We have previously suggested that part of the large loop between the first and the second putative transmembrane helices of the NQO8 subunit of *P. denitrificans* forms an amphipathic helix that is located on the internal surface of the membrane (*10*). Analyses of site-specific mutants within that region indicated that this domain may be involved in ubiquinone binding and reduction (*10*). The putative location of E158, E212, and E247 of NQO8 on the same side of the membrane as that amphipathic helix (Figure 1) and the steady-state kinetic analyses of the mutants (Table 4) may suggest that these conserved Glu residues are also involved in ubiquinone reduction. One would like to know whether these three Glu residues are located close to each other and to the putative amphipathic helix at the first conserved loop of the ND1 (or NQO8 or nuoH) protein. However, the present results could not be considered as an indication for such proximities since the ubiquinone binding site of complex I is rather spacious (*27*, *29*).

It was previously observed that *P. denitrificans* complex I mutants with low $Q_2$ reduction rates are inhibited by high acceptor concentrations (*10*). Such substrate inhibitions were not seen in the present mutants (Figure 3). This might indicate that although E158, E212, and E247 belong to the same protein and are located on the same side of the membrane as the putative surface helix in the first loop of NQO8 (*10*), they are not interacting with the same segment of the acceptor molecule.

Finally, the combined results of the present work and our previous study on this subunit (*10*) strongly suggest that amino acid residues in the NQO8 subunit which are located close to the membrane surface on the cytoplasmic side of the membrane are intimately involved in ubiquinone binding and reduction.

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