Analysis of the Pathogenic Human Mitochondrial Mutation ND1/3460, and Mutations of Strictly Conserved Residues in Its Vicinity, Using the Bacterium *Paracoccus denitrificans*†

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ABSTRACT: The human mitochondrial ND1/3460 mutation changes Ala52 to Thr in the ND1 subunit of Complex I, and causes Leber's hereditary optic neuropathy (LHON) [Huoponen et al. (1991) *Am. J. Hum. Genet. 48*, 1147]. We have used a bacterial counterpart of Complex I, NDH-1 from *Paracoccus denitrificans*, for studying the effect of mutations in the ND1 subunit on the enzymatic activity. The LHON mutation as well as several other mutations in strictly conserved amino acids in its vicinity were introduced into the NQO8 subunit of NDH-1, a bacterial homologue of ND1. The enzymatic activity of the mutants in the presence of hexammineruthenium (rotenone-insensitive) and ubiquinone-1 (rotenone-sensitive) were assayed. In addition, the kinetics of the interaction of selected mutant enzymes with ubiquinone-1, ubiquinone-2, and decylubiquinone was studied. The results suggest that the mutated residues play an important role in ubiquinone reduction by Complex I.

The H^+-translocating NADH:ubiquinone oxidoreductase is a large enzyme that is located in the mitochondrial inner membrane and in the cytoplasmic membrane of several bacteria. The mitochondrial enzyme (Complex I) is composed of some 42 different protein subunits, seven of which, ND1-ND6 and ND4L, are encoded and synthesized within the organelle (*1*, *2*). Mutations in mitochondrially encoded proteins may cause Leber's hereditary optical neuropathy (LHON).[1] In particular, each of the three primary mutations ND4/11778, ND1/3460, and ND6/14484 is sufficient for predisposition for LHON (*3*, *4*). The former two mutations were shown to slightly decrease the turnover rate of Complex I in mitochondria from cultured lymphoblasts, and the ND1/3460 mutant enzyme also exhibited increased sensitivity to inhibition by ubiquinone-2 (*5*). Thus, it was demonstrated that point mutations in the ND subunits that cause human diseases affect the activity of Complex I. However, the number of naturally occurring pathological mutations in Complex I is limited, and further studies on the role of the ND subunits in the function of the enzyme may require site-directed mutagenesis. Unfortunately, it is extremely difficult to introduce specific mutations into mitochondrially encoded proteins, such as the ND's, and one may need to find a prokaryotic system for such studies.

Bacterial Complex I (also called NDH-1) is composed of 14 subunits, seven of which are homologous to the seven ND's of the mitochondrial Complex I (*6*). NDH-1 of the soil bacterium *Paracoccus denitrificans* is probably the bacterial enzyme that is most similar to mammalian Complex I (*6*, *7*). Due to this, and to the enormous difficulties in genetic manipulation of mitochondrially encoded proteins, NDH-1 of *P. denitrificans* is an attractive model system for site-specific mutagenesis studies on the ND1 subunit of Complex I. The *nqo8* gene, encoding the ND1 of *P. denitrificans*, was previously deleted and replaced by the *ndh* gene of *Escherichia coli* that encodes NDH-2 (*8*).

The ND1/3460 mutation changes an alanine to threonine in the middle of a highly conserved region of the subunit (Figure 1). We have mutated the respective alanine in *P. denitrificans*, A65 of NQO8, as well as three strictly conserved residues in its vicinity, Q60, D64, and K67 (Figure 1). The activity of the mutants in the presence of different electron acceptors was analyzed, and the results suggest that this domain may be involved in the binding and reduction of ubiquinone.

MATERIALS AND METHODS

Materials. Ubiquinone 1 (Q_1) was a generous gift from Hofmann−La Roche, Switzerland; Ubiquinone 2 (Q_2) was a generous gift from Eisai, Japan; decylubiquinone (DB) and deamino-NADH were purchased from Sigma; and NADH was purchased from Boehringer.

Mutagenesis of P. denitrificans. Site-directed mutagenesis of cloned *nqo8* was performed according to Kunkel et al.

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[1] Abbreviations: DB, decylubiquinone (formerly decylbenzoquinone); HAR, hexammineruthenium; LHON, Leber's hereditary optic neuropathy; Q_1, ubiquinone-1; Q_2, ubiquinone-2.

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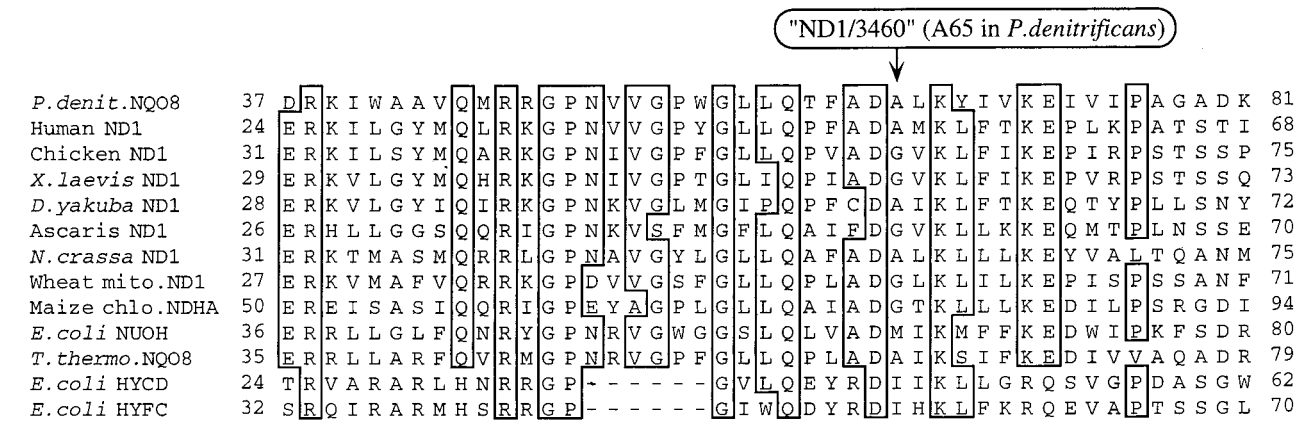

FIGURE 1: Sequence alignment of the segment between the first and second predicted trans-membrane α-helices of ND1. The position of the pathological ND1/3460 mutation is indicated. Accession numbers for the sequences shown are *P. denitrificans* NQO8, P29920; Human ND1, P03886; Chicken ND1, P18936; *X. laevis* ND1, P03890; *D. yakuba* ND1, P07710; Ascaris ND1, P24875; *N. crassa* ND1, P08774; Wheat ND1, Q01148; Maize NDHA, P25706; *E. coli* NUOH, P33603; *T. thermophilus* NQO8, Q60019; *E. coli* HYCD, P16430; *E. coli* HYFC, P77858.

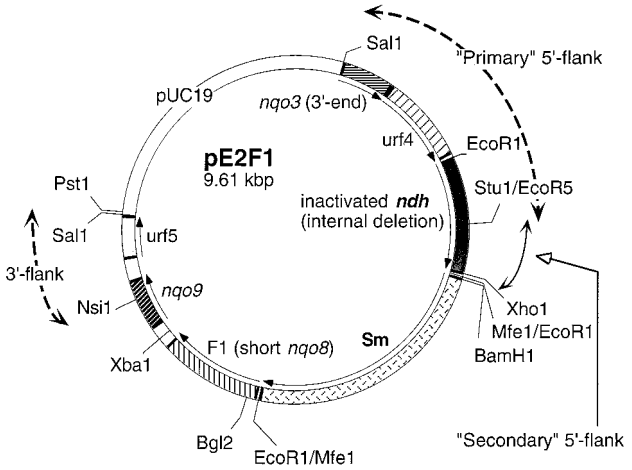

FIGURE 2: A scheme of pE2F1, and the flanks available for the incorporation of mutated *nqo8* into the *NQO* operon of Pd92-223. See text for further details.

(*9*) as modified by New England Biolabs (The NEB Transcript, December 1995), or by the Altered Sites system of Promega. The mutated genes were subcloned into pE2F1 as *Bgl*II-*Xba*I fragments (Figure 2), replacing F1 which is a shorter (inactive) version of *nqo8*. The *Sal*I fragments from the derivatives of pE2F1 (Figure 2) were subcloned into pSUP202-mp15, and the derivatives of pSUP202-mp15 were introduced into Pd92-223 by conjugation (*8*). The suicide plasmid pSUP202-mp15 was constructed by purifying the *Xho*I-*Sma*I fragment that contained the kanamycin marker from pUC-4-KIXX (Pharmacia), and the *Sma*I-*Sal*I fragment that contained *lacZ* from pMC-1871 (Pharmacia). The two fragments were simultaneously inserted into pSUP202 (*10*) that had been digested by *Sal*I. As a result, mutants that incorporated the entire pSUP202 mp15 into their chromosome by a single crossover event will appear blue when grown in the presence of X-gal, and will be resistant to kanamycin.

The *ndh* gene in pE2F1 was inactivated by digestion with *Stu*I and *Eco*RV, and ligation of the resultant blunt ends (*Stu*I/*Eco*RV in Figure 2). The incorporation of *nqo8* (and *nqo9*) back into the chromosome should occur by two crossover events, one at the 5′-flank and one at the 3′-flank of the kanamycin-resistance gene in Pd92-223 (*8*). The unmodified *ndh* gene comprises part of the 5′-flank in Pd92-223. In case crossover between the pSUP202-mp15 derivative that contained the inactivated *ndh* and the chromosome occurred within the primary flank (Figure 2), the resulting mutant will include the inactivated *ndh*. On the other hand, if crossover occurred within the secondary flank, i.e., between the *Stu*I/*Eco*RV point and the streptomycin marker (Sm, Figure 2), the mutant will express active NDH-2. Hence, white colonies of *P. denitrificans* that exhibited streptomycin resistance and kanamycin sensitivity were selected for small-scale membrane preparations in order to determine whether they express an active NDH-2 (*8*).

Membrane Isolation and Washing. Bacteria were grown in a DL-malate containing medium under high aeration in a 16-liter fermentor (*11*). Membranes were prepared by lysosyme treatment and osmotic shock (*12*) and stored at −80 °C. The membranes were further washed with a cholate-containing solution as previously described (*13*), except that phosphate buffer was used (pH 7.0) and the salt was omitted.

Activity Assays. Measurements of enzymatic activity were carried out at 30 °C in a Shimadzu UV3000 spectrophotometer. The oxidation of NADH or deamino-NADH in the presence of ubiquinone analogues or HAR was followed at 340 nm. The HAR reductase activity was measured as previously described (*15*), with minor modifications. The standard conditions for this assay were 20 mM Hepes buffer pH 8.0, 0.3 mM NADH (or deamino-NADH), 2 mM HAR, and 15 mM rotenone. The quinone reductase activities were measured in the presence of Q_1, Q_2, or DB. The reaction mixture contained 1 mg/ml sonicated soybean phospholipid, 5 mM KCN (neutralized with HCl), 50 mM KP_i pH 7.0, 0.2 mM NADH (or deamino-NADH), and a different amount of the acceptor. The latter reactions were stopped by the addition of rotenone (30 μM final concentration), and the rotenone-insensitive absorbance change was subtracted.

RESULTS

The homologue of ND1 in *P. denitrificans* is encoded by the *nqo8* gene, and a strain that lacks this gene was prepared previously (*8*). In the current study we present complementation of the deletion strain, Pd92-223, by either wild-type

Table 1: Activities of the NQO8 Mutants, and Their Dependence on NDH-2

mutant	growth without NDH-2	HAR reductase activity[a] (μmol NADH mg protein^{-1} min^{-1})	Q_1 reductase activity[b] (μmol NADH mg protein^{-1} min^{-1})	ratio of activities Q1/HAR
F2 (control)	yes	1.65 ± 0.06	0.37 ± 0.04	0.22
A65T	yes	4.25 ± 0.21	0.75 ± 0.06	0.18
A65M	yes	6.7 ± 0.6	1.02 ± 0.075	0.15
D64E	yes	4.05 ± 0.27	0.27 ± 0.018	0.067
D64N	no	1.14 ± 0.17	0.00 ± 0.00	0.00
D64C	no	0.85 ± 0.12	0.02 ± 0.001	0.024
Q60E	no	1.28 ± 0.08	0.14 ± 0.002	0.11
Q60G	no	1.9 ± 0.046	0.04 ± 0.003	0.02
K67A	no	0.87 ± 0.014	0.06 ± 0.003	0.07
K67E	no	0.10 ± 0.005	0.002[c]	0.02[c]

[a] Activity values are averages of at least three measurements (± SD). [b] The activity was determined in the presence of 60 mM Q_1. [c] The turnover rate of K67E in the presence of Q_1 was barely above the detection limit, and hence it should be viewed with caution.

(F2) or mutated *nqo8* that was inserted back into the *NQO* operon. The complementation was designed to yield NDH-1 mutant strains that lack NDH-2 activity. The latter was introduced into Pd92-223 in order to allow deletion of *nqo8* and *nqo9* (*8*), but its activity hampers analysis of the proton translocation activity of the mutants when intact cells are used. On the other hand, poorly active NDH-1 mutants would not grow without an active NDH-2. Due to this we have developed a complementation system that allows inactivation of NDH-2 in cases where the activity of the mutant NDH-1 is sufficient to support bacterial growth (see Materials and Methods).

We have introduced the ND1/3460 mutation into the *nqo8* gene of *P. denitrificans*, generating A65T. In addition, the *E.coli* version of this site was prepared (A65M), and three strictly conserved amino acids in the vicinity of A65, namely Q60, D64, and K67 (Figure 1) were replaced by different residues (Table 1). The mutated *nqo8* genes were incorporated into the chromosome of the deletion strain, Pd92-223, and colonies with the correct phenotype (i.e., white, streptomycin-resistant and kanamycin-sensitive) were assayed for the presence of active NDH-2. In most cases all the colonies from a given mutant exhibited a similar pattern of NDH's; i.e., either all contained or all lacked NDH-2. However, in some cases two types of colonies were found. An interesting mutant in this respect is D64E since three out of the six colonies that were resistant to streptomycin and sensitive to kanamycin contained NDH-2, while the other three lacked such activity. The latter type of D64E strain was used for large-scale membrane preparations and for the different analyses described below.

The absence of NDH-2 from the F2 (control), A65T (LHON), and D64E mutants enabled examination of their H^+-translocation activity. This was assayed by pulsing a suspension of anaerobic cells with air-saturated water (*14*), and the results indicate that the A65T and D64E mutations do not affect the proton translocation efficiency of the enzyme (not shown).

The specific NADH oxidase activity of the mutants with HAR (rotenone-insensitive) and Q_1 (rotenone-sensitive) as electron acceptors are presented in Table 1. HAR turned out to be more suitable for such analyses than the widely used ferricyanide. The HAR reductase activity of NDH-1

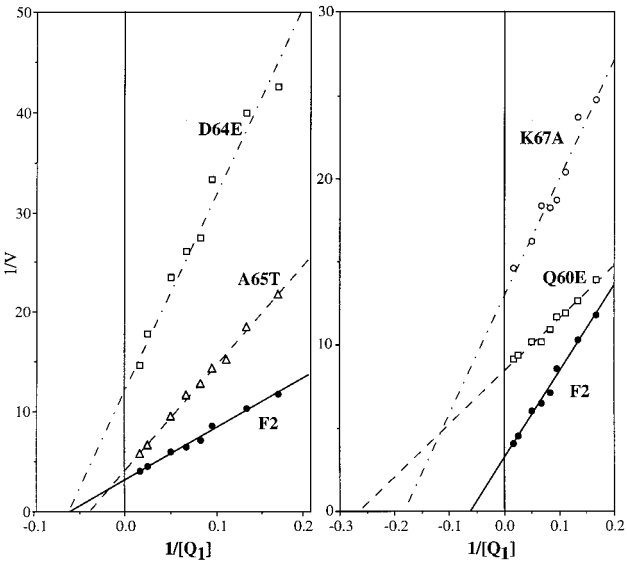

FIGURE 3: Lineweaver−Burk analysis of the kinetics of NADH (or deamino-NADH) oxidation by selected mutants in the presence of Q_1. F2 is the control (complemented with wild-type gene). The mutants that grew without NDH-2 are shown on the left, and those with active NDH-2 (except the control) on the right. The units of 1/[acceptor] are mM^{-1}.

of *P. denitrificans* is higher than its ferricyanide reductase activity (Zickermann and Finel, unpublished results), and it exhibits no substrate inhibition at high concentration of either HAR or NADH (*15*). The ubiquinone reductase activity of the mutants described here was sensitive to rotenone. The sensitivity of Complex I or NDH-1 to this inhibitor is easily lost upon enzyme purification, and hence the rotenone-sensitivity of the mutant enzymes indicates that the mutations did not cause extensive structural changes in the hydrophobic segment of the enzyme. The gross structural integrity of the hydrophilic segment of the mutants was shown by electron paramagnetic resonance spectroscopy (EPR). Samples of concentrated membrane extract from F2 and mutants D64E, D64C, A65T, Q60E, and K67A were prepared and reduced with NADH or deamino-NADH. The results indicate that the *g*-values of the more easily detectable Fe-S cluster N-1b and N-2 were unaffected by the mutations (not shown).

The HAR reductase activity of Complex I (and NDH-1) does not exhibit substrate inhibition by NADH, and it only involves the hydrophilic segment of the enzyme (*15*). Due to this it may be used to estimate the concentration of assembled NDH-1 in a given sample of washed membranes. The relative rates of the rotenone-sensitive Q_1 reductase and HAR reductase activities (the ratio of activities, Table 1) is hence a measure of the specific rotenone-sensitive enzyme turnover velocity. Interestingly, the membranes of mutants A65T, A65M, and D64E contained high concentration of NDH-1, but their specific turnover with Q_1 as acceptor was lower than in the control (Table 1). The relatively high NDH-1 concentration in cells carrying the A65T, A65M, and D64E mutations, all of which lack active NDH-2 (see above), might indicate that *P. denitrificans* can regulate the expression level of NDH-1 in response to the $NADH/NAD^+$ ratio in the cytoplasm, or the ubiquinol/ubiquinone ratio in the membrane.

Mutations at the D64 position had a dramatic effect on the specific Q_1 reductase activity (Table 1). Even the mildest

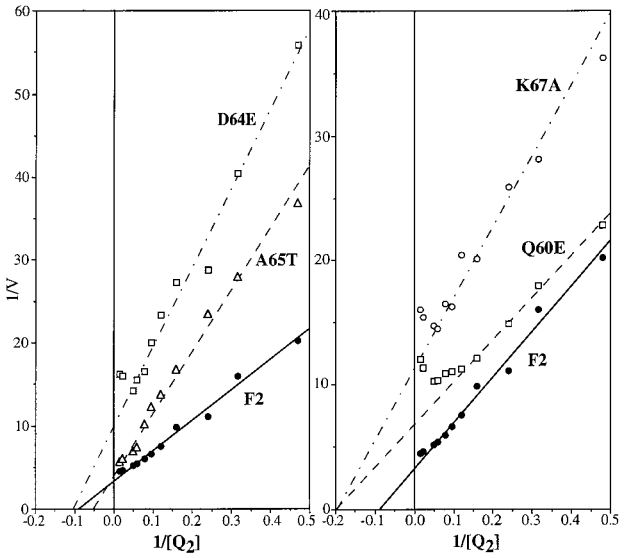

FIGURE 4: Like Figure 3, except that the acceptor was Q_2.

Table 2: Kinetic Parameters for Q_1, Q_2, and DB Reductase Activities of Selected NDH-1 Mutants

	Q_1		Q_2		DB	
mutant	V_{max}[a]	K_m (μM)	V_{max}[a]	K_m (μM)	V_{max}[a]	K_m (μM)
F2 (control)	0.32	16.5	0.31	11.1	0.28	67.5
A65T	0.25	26.2	0.26	18.7	0.25	127.0
D64E	0.08	16.5	0.10	9.7	0.13	81.0
Q60E	0.12	3.7	0.15	5.0	0.12	41.6
K67A	0.08	5.4	0.09	5.1	0.11	83.0

[a] The V_{max} values are relative to the turnover rate in the presence of HAR.

change, D to E, caused a pronounced decline in the rate of electron transfer to ubiquinone, and mutagenesis to asparagine abolished the quinone reduction activity (Table 1). Interestingly, mutant D64C had a low but measurable Q_1 reductase activity (Table 1).

Mutations at the Q60 and K67 positions exhibited a clear effect on the quinone reductase activity, the extent of which was strongly dependent on the replacing residue. Changing Q60 to glutamic acid was apparently less detrimental for the activity than replacement with glycine (Table 1).

The kinetics of NADH oxidation in the presence of Q_1, Q_2, and DB was examined in the control (F2) and mutants A65T, D64E, Q60E, and K67A (Figures 3 and 4, and Table 2). The LHON mutant, A65T, had a higher apparent K_m than F2 for the three quinone analogues, and its V_{max} was about 80% of the control (Table 2 and Figures 3 and 4). Mutant D64E had a rather low V_{max} in the presence of all these acceptors. However, the apparent K_m of D64E in the presence of all the three ubiquinones was similar to the respective values in F2. Mutants Q60E and K67A exhibited low V_{max} values, but their apparent K_m for Q_1 and Q_2 were significantly lower than the control (Table 2 and Figures 3 and 4). Substrate inhibition by Q_2 was detectable in mutants Q60E, D64E, and K67A (Figure 4). Substrate inhibition by either Q_1 (Figure 3) or DB (not shown) was not observed in these mutants, nor in A65T.

DISCUSSION

We have developed a unique complementation system for NQO8 of *P. denitrificans*. Its main feature is the possibility to isolate cells that have active NDH-1 mutants and lack NDH-2, as well as inactive NDH-1 mutants that express the alternative enzyme. This system eliminates selection pressure on poorly active mutants, and hence drastically reduces the risk that such mutants will mistakenly appear as active ones in cases where only second-site revertants would grow.

The ubiquinone binding site of Complex I was previously suggested to be in the ND1 subunit, but experimental support for this has been inconclusive. The ND1 subunit was reported to be labeled by rotenone (*16*). However, since rotenone is a noncompetitive inhibitor (*17*), its binding site is not necessarily an indication for a ubiquinone binding site. Another suggestion was based on sequence homology between ND1 and the piericidin A-sensitive enzyme glucose oxidase (*18*), but this homology is probably too low to be meaningful (*1*). The increased sensitivity of Complex I from mitochondria carrying the ND1/3460 mutation to inhibition by Q_2 was also taken to suggest that ND1 is involved in ubiquinone binding (*5*).

In the present work we have shown that mutations in specific amino acids of the NQO8 subunit of NDH-1 from *P. denitrificans* decrease or even abolish the specific ubiquinone reductase activity of the enzyme (Table 1). In addition, the LHON mutation (A65T) significantly raises the apparent K_m for the three ubiquinone analogues, while the respective V_{max} values are decreased by about 20% (Figures 3 and 4, and Table 2). This strongly suggests that the A65T mutation specifically interferes with the binding of ubiquinone. In this respect it is interesting that mutants D64E, K67A, and in particular Q60E exhibited sensitivity to high concentration of Q_2, a ubiquinone analogue that serves as a good electron acceptor from the wild-type and the other NDH-1 mutants (Figure 4 and Table 2). Hence, our results provide new and independent indications that the ND1 subunit, or its bacterial homologue NQO8, is directly involved in the ubiquinone reductase activity of Complex I. It might be argued that the mutations affected the ubiquinone reductase activity indirectly, by altering the assembly of the membrane domain of NDH-1. However, the presence of rotenone-sensitive ubiquinone reductase activity renders such a possibility unlikely, at least as far as the A65T, A65M, D64E, Q60E, and K67A mutants are concerned.

We have identified a specific domain and amino acids within the NQO8 subunit of NDH-1, i.e., the segment around A65, that play an important role in the binding and reduction of ubiquinone by the enzyme. Secondary structure prediction suggests that this segment forms an α-helix, and a helical wheel analysis of the putative helix shows clear amphipathy (Figure 5). All the highly conserved residues of this stretch, most of which are polar, fall on one side of the helix, while the other side contains only hydrophobic amino acids (Figure 5). Due to this, and to its location in a loop between two predicted trans-membrane α-helices, it is suggested that this domain may be a helix on the surface of the membrane. According to this model residues K67 and Q60 are pointing to the opposite direction than A65 (Figure 5). Interestingly, both mutants K67A and Q60E exhibited lowered apparent K_m values for Q_1, while A65T exhibited an elevated apparent K_m for this substrate (Table 2).

The D64E mutation affected the V_{max} values but not the apparent K_m for the different acceptors (Table 2 and Figures 3 and 4), suggesting that D64 plays a role in ubiquinone

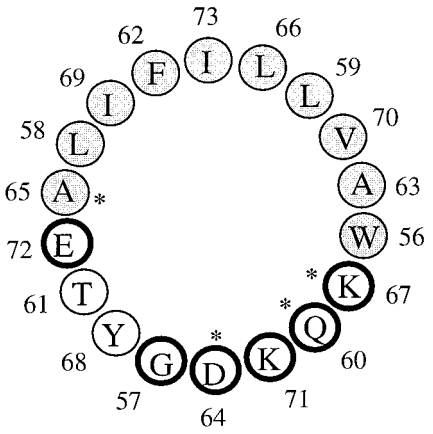

FIGURE 5: Helical wheel presentation of the segment surrounding A65 in NQO8 of *P. denitrificans*. Highly conserved residues are indicated by a bold circle, and hydrophobic residues are lightly shaded. The asterisks indicate amino acids that were mutated in this study.

reduction rather than its binding. It is tempting to speculate that this mutation may alter the protonation rate of a bound ubisemiquinone by protons from the aqueous phase. In any case, the low rate of Q_1 reduction in the D64C mutant enzyme, and the apparent absence of such activity in the case of D64N (Table 1), suggests that an acidic amino acid at this position is essential for ubiquinone reduction. In this respect it may be noted that mutant E286C of the H^+-translocating terminal oxidase of *E. coli*, cytochrome bo_3 is much more active than mutant E286Q (*19*).

The ubiquinone reductase activity of mitochondrial Complex I is inhibited by the acceptor Q_2 (*20*), and the ND1/3460 mutation increased the sensitivity of the human enzyme to inhibition by this substrate (*5*). At the outset of this study it was anticipated that the NQO8/A65T mutation would affect the NDH-1 of *P. denitrificans* in a similar way. However, the V_{max} of this mutant enzyme was similar in the presence of all the three quinones (Table 2), and no substrate inhibition by Q_2 was observed (Figure 4). The reason for this may be related to the observation that NDH-1 of *P. denitrificans*, in contrast to Complex I from bovine and human mitochondria, is not inhibited by Q_2 (Figure 4; and Zickermann and Finel, unpublished results). Mutants Q60E, D64E, and K67A were sensitive to inhibition by Q_2 (Figure 4), but it remains to be established whether the mechanism of this substrate inhibition is similar to that in mitochondrial Complex I.

The results of this work may shed new light on the effect of the ND1/3460 mutation on the activity of Complex I in LHON patients. The homologous mutation in NDH-1, A65T, does not change the stoichiometry of proton translocation by the enzyme, but affects the kinetic properties of ubiquinone reduction. The V_{max} of the LHON mutant in NDH-1 is about 80% of the control (Table 2), a similar change to that observed in human mitochondria (*5*). The small but significant effect of this mutation on the apparent K_m for the short-chain ubiquinones that was revealed by this study (Table 2), may suggest that Complex I of the LHON patients has an increased K_m for ubiquinone-10. Such a K_m change might decrease the in vivo activity of the enzyme below the threshold that is required for normal function of the optic nerve in adults.

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