The Proton-translocating NAD-Quinone Oxidoreductase (NDH-1) of Thermophilic Bacterium *Thermus thermophilus* HB-8

**COMPLETE DNA SEQUENCE OF THE GENE CLUSTER AND THERMOSTABLE PROPERTIES OF THE EXPRESSED NQO2 SUBUNIT**

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The genes encoding the proton-translocating NADH-quinone oxidoreductase (NDH-1) of a thermophilic bacterium *Thermus thermophilus* HB-8 were cloned and sequenced. They constitute a cluster that is composed of 14 structural genes and contains no unidentified reading frames. All of the 14 structural genes, which are designated NQO1–14, encode subunits homologous to those of *Paracoccus denitrificans* NDH-1, respectively, and are arranged in the same order as other bacterial NDH-1 genes. *T. thermophilus* NDH-1 contains at least nine putative iron-sulfur cluster binding sites, eight of which are commonly found in other organisms. The *T. thermophilus* NQO2 subunit was expressed in *Escherichia coli*. The expressed subunit bears a single [2Fe-2S] cluster whose optical and EPR properties are very similar to those of N1a cluster in the *P. denitrificans* NQO2 subunit (Yano, T., Sled', V.D., Ohnishi, T., and Yagi, T. (1994) *Biochemistry* 33, 494–499). These results strongly suggest that the *T. thermophilus* NDH-1 is similar to other NDH-1 enzyme complexes in terms of subunit and cofactor composition. The *T. thermophilus* NQO2 subunit displayed much higher stability than the mesophilic equivalent and its iron-sulfur cluster remained intact even after incubation for 3 h at 65 °C under anaerobic conditions. With the advantage of thermostability, the *T. thermophilus* NDH-1 provides a great model system to investigate the structure-function relationship of the NDH-1 enzyme complexes.

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NADH-quinone oxidoreductase (Complex I) of the mitochondrial respiratory chain is a membrane-bound enzyme complex that catalyzes the oxidation of NADH with ubiquinone as an electron acceptor. This enzyme complex concomitantly pumps protons across the inner membrane to generate an electrochemical proton gradient by utilizing energy obtained from the redox reaction as a driving force. Mitochondrial Complex I has been extensively studied using the bovine heart enzyme (1, 2). Complex I is composed of at least 41 dissimilar subunits, and its molecular mass is estimated to be more than 900 kDa. Complex I contains one non-covalently bound FMN and several iron-sulfur clusters as redox components. Some of the latter are EPR-visible and are designated N1a and N1b (for binuclear clusters) and N2, N3, and N4 (for tetranuclear clusters).

Recently, bacterial proton-translocating NADH-quinone oxidoreductase (NDH-1) has emerged as a useful and simpler model system for the study of Site I energy coupling (3). NDH-1 enzyme complexes have been isolated and characterized from several bacteria such as *Paracoccus denitrificans* and *Escherichia coli* (4, 5). The bacterial NDH-1 is a multiple-subunit enzyme complex and contains one non-covalently bound FMN and several iron-sulfur clusters similar to the mitochondrial Complex I. Genes encoding the NDH-1 were first cloned and sequenced from *P. denitrificans* (6–10). The genes constitute a cluster that is composed of 14 structural genes (designated NQO1–14) and 6 unidentified reading frames (URFs). All of the 14 subunits have mitochondrial homologues; seven subunits correspond to promontory, nuclear encoded subunits, and seven correspond to the hydrophobic mitochondrial encoded subunits, the so-called ND gene products (11). The *P. denitrificans* NDH-1 conserves all putative cofactor binding sites, suggesting that mitochondrial Complex I and NDH-1 share a similar structure and functional mechanism (3, 12). Recently, complete DNA sequences of *E. coli* NDH-1 and partial sequences of *Salmonella typhimurium*, *Rhodobacter capsulatus*, and *Synechocystis sp.* (PC06803) NDH-1 have been reported (13–16). Those sequences have shown the same structural features, providing further support that the bacterial NDH-1 is akin to the mitochondrial Complex I.

*Thermus thermophilus* strain HB-8, which was isolated from a hot spring in Japan, is an extremely thermophilic, obligatory aerobic, Gram-negative, and chemoheterotrophic bacterium (17). The bacterium is capable of growing in the temperature range of 45–85 °C with optimal growth temperature of 70 °C. It was suggested that its respiratory chain may include energy flavoprotein fraction; IP, iron-sulfur protein fraction; URF, unidentified reading frame; *Tbr*, *T. bryantii*; *Th*, *T. thermophilus*; *Pd*, *P. denitrificans*; *Ee*, *E. coli*; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

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coupling Site I (18). Later, it was shown that T. thermophilus HB-8 possesses two types of NADH-quinone oxidoreductase (19). One is a proton-translocating NADH-dehydrogenase (NDH-1), and the other is a small non-energy-coupled enzyme referred to as NDH-2. Both enzymes have been purified and characterized to some extent. The NDH-1 was found to be composed of at least 10 dissimilar polypeptides and contained one non-covalently bound FMN, 11–12 mol of non-heme iron and 7–9 mol of acid-labile sulfide/mol. The NDH-2 was shown to be a single polypeptide with non-covalently bound FAD and no iron-sulfur clusters. Both enzymes can utilize NADH and deamino-NADH as substrates (in contrast to mesophilic NDH-2) and catalyze NADH-oxidation with water-soluble electron acceptors such as K$_2$[Fe(CN)$_6$] and Q$_A$ at high temperature (≥80 °C) (19). More recently, iron-sulfur clusters of the membrane-bound T. thermophilus HB-8 NDH-1 enzyme complex have been studied by EPR spectroscopy. At least three iron-sulfur clusters (one binuclear and two tetranuclear clusters) and possibly two additional iron-sulfur clusters (one binuclear and one tetranuclear) with very low redox mid-point potentials were detected in NDH-1 (20). Notably, cluster N2 signals were not seen in the study. We expect that the thermostability will provide a great advantage for structural studies of the NDH-1 either by NMR or x-ray crystallography. Furthermore, since T. thermophilus contains only menaquinone, it will provide an interesting system for understanding the Site I energy coupling mechanism.

This paper reports molecular cloning, sequencing, and expression studies of the proton-translocating NADH-quinone oxidoreductase (NDH-1) of T. thermophilus HB-8. The genes encoding the T. thermophilus NDH-1 enzyme complex constitute a gene cluster that is composed of 14 structural genes (designated NQO1–14) and no URFs. All of the 14 structural genes encode subunits homologous to the P. denitrificans NDH-1 enzyme complex. Comparison of the deduced amino acid sequences with those of other organisms revealed that T. thermophilus NDH-1 possesses principally the same molecular structure as other bacterial NDH-1 in terms not only of subunit composition but also of cofactor binding sites. The NQO2 subunit of T. thermophilus NDH-1 was expressed in E. coli, purified, and partially characterized.

**EXPERIMENTAL PROCEDURES**

**Genomic DNA Preparation—**T. thermophilus HB-8 (ATCC27634) was grown in Thermus nutrient medium at 70 °C to late exponential phase according to Hon-nami and Oshima (21). The cells were harvested by centrifugation in a SS34 rotor at 6,000 rpm for 10 min. The cells were washed once with 10 ml Tris-HCl (pH 8.0) containing 0.1 M NaCl and 0.1 mM EDTA and resuspended in 2.0 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM EDTA (TE buffer). The cell suspension was then treated with 3 mg/ml lysozyme at 42 °C for 20 min. Twelve ml of 0.1 M Tris-HCl (pH 8.0) containing 0.1 M NaCl and 1% (w/v) SDS were added, and the cell suspension was freeze-thawed three times with liquid nitrogen and a 80 °C water bath. DNA was extracted with 14 ml of phenol/chloroform/TE for 10 min, followed by centrifugation three times. The aqueous phase was carefully transferred and dialyzed against 1 liter of 50 mM Tris-HCl (pH 8.0) containing 10 mM NaCl overnight with one buffer change. The DNA solution was incubated with 100 μg/ml RNase A at 37 °C for 3 h and then with 100 μg/ml proteinase K and 0.1% (w/v) SDS at 50 °C for 2 h. The solution was successively treated with an equal volume of phenol/chloroform/TE and then with chloroform/isoamyl alcohol. DNA was collected with a glass rod after the addition of 2.5 volumes of cold ethanol and suspended in TE buffer.

Cloning and Sequencing of the T. thermophilus NDH-1 Genes—To date, two complete (P. denitrificans and E. coli) and three partial DNA sequences (S. typhimurium, R. capsulatus, and Synechocystis) of the NDH-1 enzyme complexes have been deduced and are available in GenBank™ (6–10, 13–16). In the two bacteria, the NDH-1 genes are organized as a gene cluster. Therefore, it could be anticipated that T. thermophilus HB-8 NDH-1 genes constitute a gene cluster. Hence, no amino acid sequence information was available in T. thermophilus NDH-1, we decided to amplify DNA fragments by polymerase chain reaction (PCR) with oligonucleotides designed on the basis of sequence similarities between organisms whose sequences had already been determined. The NDH-binding subunit of T. thermophilus NDH-1 was previously identified as the 47-kDa subunit by gel electrophoresis with [3H]NADH, and its amino acid composition was found to be very similar to those of P. denitrificans NQO1 and bovine heart Complex I (14). The 47-kDa subunit, N. crassa 51-kDa subunit, E. coli NQOF, and S. typhimurium NQOF: TT1F (sense), 5′-ATC TCG (CT) GGA (AG/C/T) GAA (AG/G) CA-3′; TT2F (sense), 5′-AAG CCT (AG/C/T) CCA (AG/G/C/T) CCT (CA/G/C) GC-3′. GC-3′ from K244PPAPA46; TT3R (antisense), 5′-TCC (G/C) CGT (AG/G/C/T) GAA (AG/G) CA (AG/G/C/T) GCA (AG/G) CA-3′ (Duarte et al. (23)). The 47-kDa subunit was subsequently obtained as five clones that were designated as T. thermophilus HB-8 genomic DNA, and 2.5 units of Taq polymerase (Life Technologies, Inc.). The amplification was performed in a thermostylic with a reaction program that consisted of one cycle of hot start (94 °C for 10 min, 60 °C for 5 min), 35 amplification cycles that were composed of denaturing (94 °C for 1 min), annealing (50 °C for 1.5 min), and elongation (72 °C for 2 min), and finally one cycle of termination (72 °C for 10 min). Amplified DNA was purified with QiAquick PCR purification kit (Qiagen) and run on a 1.0% (w/v) agarose gel. The DNA fragments of desired molecular sizes were excised and purified from gel with QiAquick gel extraction kit and then ligated with Taq polymerase (Life Technologies, Inc.). The amplification was performed in a thermostylic with a reaction program that consisted of one cycle of hot start (94 °C for 10 min, 60 °C for 5 min), 35 amplification cycles that were composed of denaturing (94 °C for 1 min), annealing (50 °C for 1.5 min), and elongation (72 °C for 2 min), and finally one cycle of termination (72 °C for 10 min). Amplified DNA was purified with QiAquick PCR purification kit (Qiagen) and run on a 1.0% (w/v) agarose gel. The DNA fragments of desired molecular sizes were excised and purified from gel with QiAquick gel extraction kit (Qiagen) and then subcoloned into TA cloning vector based on pBluescript KS+ (Stratagene) that had been prepared according to Papp et al. (23). When two sets of oligonucleotides, TT1F (sense)/TT3R (antisense) and TT2F (sense)/TT3R (antisense), were used as primers, DNA fragments with molecular sizes of 540 and 480 bp were specifically amplified by PCR, respectively (data not shown). The 540-bp fragment contained an identical 540-bp DNA fragment. When the putative amino acid sequence derived from 540-bp DNA fragment was compared with those of the NDH-binding subunits of other organisms, a high degree of sequence similarity was found. Therefore, we concluded that the amplified DNA fragments encoded a part of the T. thermophilus HB-8 NDH-binding subunit and could be used as a probe to clone the gene cluster. The standard cloning techniques used were essentially those of Sambrook et al. (24). Southern-blot hybridization was performed with DNA probes non-radioactively labeled with Genius 1 DNA labeling and detection kit (Boehringer Mannheim) according to the manufacturer’s protocol. With the 540-bp DNA fragment as the first probe, a 5.5-kilobase pair HindIII fragment was cloned (designated as pTTH-1) from a mini library constructed from 5.0–6.0-kilobase pair HindII fragments digested from genomic DNA of T. thermophilus HB-8. The clone contained genes that were homologous to the downstream half of the P. denitrificans NQO1, the entire region of the P. denitrificans NQO2, NQO1, and NQO3 and the upstream part of the P. denitrificans NQO8 (Fig. 1). DNA encompassing the entire T. thermophilus NDH-1 gene cluster was subsequently obtained as five clones that were designated as pTTH–1, respectively, as shown in Fig. 1. DNA sequencing was performed using the dideox-chain termination method with the Applied Biosystems dye terminator cycle sequencing kit and T3, T7, and synthetic unique oligonucleotide primer. The DNA sequence of total 14,886 bp (from SacII to BamHI in Fig. 1) and the predicted amino acid sequences were deposited in GenBank under an accession number of TAA52917.

**Sequence Analysis—**GCAT software programs were used to analyze the DNA and amino acid sequences (25). Sequence comparison of the polypeptides were conducted with BESTFIT and PILEUP programs. The FASTA and PROFILESEARCH programs were used to search the GenBank/EMBL sequence data bases for proteins having some homology to the polypeptides. Homology search was also carried out by using the BLAST program running at the National Center for Biotechnology Information. Coding regions were searched with CODONPREF-
ERENCE, and hydrophobic profiles were examined with HYDROPLOT.

Construction of an Expression Vector for the T. thermophilus NQO2 (TthNQO2) Subunit—The NQO2 gene was obtained from the pTTH-1 plasmid by PCR using two oligonucleotides to introduce the same buffer. The column was washed with 50 ml of degassed 50 mM containing 1.0 mM PMSF, and 20 ml of para cell disruption bomb (nitrogen and a 30°C water bath) and were then passed twice through a Parr cell disruption bomb (28). SDS-polyacrylamide gel electrophoresis was carried out by a modified method of Laemmli (29). Immunoblotting was conducted as described previously (30–32). Non-heme iron and acid-labile sulfide were determined according to Doeg and Ziegler (33) and Fogo and Popowski (34), respectively. DNA sequences from the procedures were determined according to Sanger et al. (35). Any variations from the procedures or other details are described in the figure legends.

Materials—Acrylamide, N,N'-methylenebis(acrylamide), SDS, SDS-PAGE marker proteins, and Coomassie Brilliant Blue R-250 were from Bio-Rad (Hercules, CA) and horseradish peroxidase-conjugating affinity-purified antibodies to rabbit IgG were from Calbiochem; expression vectors, pET16b, and E. coli strain BL21(DE3)pLysS were from Novagen. All other chemicals were of the highest grade available from Sigma.

RESULTS

Structural Properties of the Gene Cluster—One of the unusual features of the T. thermophilus NDH-1 genes is their very high G+C content (an average of 68.3%). High preference of G+C in the third codon position (93.9%) results in a unique codon usage (17, 36). In order to identify the reading frames of the cloned DNA, we utilized (i) a codon preference table that had been built on the basis of 45 T. thermophilus DNA coding sequences reported in GenBank™, (ii) amino acid sequence similarities to homologous subunits of other organisms (human, bovine heart, N. crassa, P. denitrificans, E. coli, S. typhimurium, R. capsulatus, etc.), and (iii) relative distance of putative translation initiation sites to ribosome binding site (Shine-Dalgarno sequence) (37). It was found that the cluster is composed of 14 open reading frames and contains no URFs (Fig. 1 and Table I). Five structural genes utilize unique translation start codons. GTG start codons were predicted in NQO6, NQO8, NQO11, and NQO13, and TTG start codon in the NQO7 gene (Table I). To our knowledge, nine genes have been found to utilize GTG start codon out of 45 T. thermophilus DNA sequences in GenBank™. However, no TTG start codon has been reported to date in T. thermophilus. These rare start codons should be considered as tentative, and the actual translation start positions of the open reading frames must be experimentally identified in the future. The NDH-1 gene cluster contains a promoter-like sequence in its upstream region. The sequence 5'-CCCCCTGGCG-3' and 5'-TAAGAT-3' are homologous to those of promoter regions of other T. thermophilus genes that have been empirically studied (38). These sequences are tentatively assigned to be −35 and −10 boxes, respectively. The gene cluster ends with a stem-loop-like structure that starts 49 bp downstream of the NQO14 stop codon. In addition, all 14 structural genes are so compactly organized in the cluster that there is almost no intergenic space where promoter or terminator-like sequences can be present. These properties strongly suggest that the T. thermophilus NDH-1 genes constitute an operon.

Predicted Amino Acid Sequence of Individual Subunits of the T. thermophilus NDH-1—The deduced primary sequences of the T. thermophilus NDH-1 subunits were compared with those of other organisms (e.g. bovine heart, N. crassa, P. denitrificans, and E. coli) and with those of some phylogenetically related enzymes (e.g. Alcaligenes eutrophus NAD−-reducing hydrogenase, E. coli formate hydrogen lyase). All of the 14 subunits of the T. thermophilus NDH-1 are homologous to their mitochondrial and bacterial counterparts. Here, several important and interesting features of individual subunits are described in conjunction with the latest information of mitochondrial Complex I and bacterial NDH-1.

TthNQO1 Subunit—The TthNQO1 subunit is homologous to bovine FP 51-kDa PdoNQO1, and EcNuoF subunits (Fig. 2 and Table I). This subunit plays an essential role in initiating the redox reaction by oxidizing NADH. As described above, the 47-kDa subunit was photoaffinity-labeled by [32P]NAD(H), indicating that the NDH-binding site is located in the subunit (22). The TthNQO1 subunit conserves a typical nucleotide binding sequence motif (G64XG66XG106) with remote acidic amino acid residues D84E95. A tetranuclear iron-sulfur cluster
(N3) is thought to be located in the NADH-binding subunits (39, 40). The TthNQO1 subunit contains five conserved cysteine residues (Cys182, Cys353, Cys356, Cys359, and Cys400). Four of them form a cluster with a typical motif for a [4Fe-4S] cluster binding site (C353XXC356XXC359), and Cys400 is followed by Pro (Fig. 2). Therefore, Cys400 seems more likely to be the fourth ligand residue of the [4Fe-4S] cluster than Cys182. The Tth-NQO1 subunit and its homologues are also thought to accommodate an FMN that is an indispensable component for NADH oxidation (40). As discussed in previous papers (2, 6, 41), however, it is difficult to predict the FMN-binding site on the basis of sequence data at the present time mainly due to lack of consensus sequence motifs for this cofactor.

**TthNQO2 Subunit**—The TthNQO2 subunit is homologous to bovine FP 24-kDa, PdNQO2, and EcNuoE subunits (Table I). Expression studies of the PdNQO2 subunit have shown that this subunit bears a single [2Fe-2S] cluster, which is tentatively assigned as cluster N1a (26). Further mutagenesis studies of PdNQO2 subunit have indicated that the [2Fe-2S] cluster is coordinated by four conserved cysteine residues (Cys96, Cys101, Cys137, and Cys141, *P. denitrificans* numbering) (42). The TthNQO2 subunit also conserves these four cysteine residues (Cys83, Cys88, Cys124, and Cys128).

**TthNQO3 Subunit**—The TthNQO3 subunit is homologous to bovine IP 75-kDa, PdNQO3, and EcNuoG subunits (Fig. 3 and Table II). This subunit generally contains 12 invariant cysteine residues in the N-terminal region that are possible iron-sulfur cluster ligands (8, 14, 43). Recently, expression studies of the PdNQO3 subunit showed that the subunit contains multiple iron-sulfur clusters: one [2Fe-2S] cluster (N1b), one [4Fe-4S] cluster (N4), and possibly another [4Fe-4S] cluster (44). Interestingly, it was found that the TthNQO3 subunit conserves...
only 11 cysteine residues (Cys34, Cys45, Cys48, Cys83, Cys119, Cys122, Cys181, Cys184, Cys187, and Cys230), as the 12th is replaced by Val (Fig. 3). It is worth noting that this cysteine residue is also absent from the HoxU subunit of *A. eutrophus* NAD\(^1\)-reducing hydrogenase (Fig. 3) (45). Assuming three iron-sulfur clusters (one binuclear and two tetranuclear clusters) in the subunit, this implies that at least one of the clusters has to utilize a non-cysteinyl ligand residue.

| T. thermophilus NDH-1 | vs. bovine Complex I | vs. P. denitrificans NDH-1 | vs. E. coli NDH-1 |
|----------------------|----------------------|---------------------------|------------------|
| NQO1                 | 46.0% (FP 51-kDa)\(^a\) | 43.6% (NQO1)               | 47.0% (NUOF)     |
| NQO2                 | 33.7% (FP 24-kDa)     | 34.3% (NQO2)               | 28.4% (NUOE)     |
| NQO3                 | 30.4% (IP 75-kDa)     | 30.4% (NQO3)               | 28.2% (NUOG)     |
| NQO4                 | 44.4% (IP 49-kDa)     | 42.6% (NQO4)               | 40.6% (NUOD)     |
| NQO5                 | 27.8% (IP 30-kDa)     | 33.0% (NQO5)               | 39.6% (NUOC)     |
| NQO6                 | 52.8% (PSST)          | 52.4% (NQO6)               | 48.0% (NUOB)     |
| NQO7                 | 30.6% (ND3)           | 40.0% (NQO7)               | 31.6% (NUOA)     |
| NQO8                 | 42.2% (ND1)           | 41.0% (NQO8)               | 47.7% (NUOH)     |
| NQO9                 | 42.1% (TYKT)          | 39.2% (NQO9)               | 45.0% (NUO)      |
| NQO10                | 19.5% (ND6)           | 31.4% (NQO10)              | 31.6% (NUOJ)     |
| NQO11                | 30.4% (ND4L)          | 44.1% (NQO11)              | 33.7% (NUOK)     |
| NQO12                | 34.0% (ND5)           | 44.8% (NQO12)              | 45.3% (NUOL)     |
| NQO13                | 28.5% (ND4)           | 34.0% (NQO13)              | 30.7% (NUOM)     |
| NQO14                | 27.8% (ND2)           | 35.4% (NQO14)              | 39.4% (NUON)     |

\(^a\) Respective counterparts are indicated in parentheses.
typical sequence motif for a [4Fe-4S] cluster (C181 XX C184 XX C187 ... C230P231, T.thermophilus numbering). Although there is no typical binding sequence motifs for a binuclear iron-sulfur cluster, preliminary results of resonance Raman spectroscopic studies of the expressed PdNQO3 subunit have suggested that the [2Fe-2S] cluster (N1b) is coordinated by cysteinyl residues only.3 If another cluster, [4Fe-4S], is present, it must utilize a non-cysteinyl ligand within a novel binding motif. Furthermore, the TthNQO3 subunit contains an additional cysteine cluster (C256 XX C259 XXX C263 ... C291) that is also found in E. coli and S.typhimurium NuoG subunits but not in other NDH-1 enzyme complexes (Fig. 3). It has been reported that the E. coli NDH-1 enzyme contains an additional EPR detectable [2Fe-2S] cluster (three [2Fe-2S] clusters are designated as N1a, N1b, and N1c), although the exact location of the third [2Fe-2S] cluster is not known (5, 46). Thus, it is of interest to investigate if another [2Fe-2S] cluster is located in the TthNQO3 subunit and, if so, what role it plays in electron transfer. In order to address these issues, expression of the TthNQO3 subunit is in progress in our laboratory.

TthNQO4 and TthNQO5 Subunits—
The TthNQO4 and Tth-NQO5 subunits are homologous to the bovine IP 49-kDa, Pd-NQO4, and Ec-NuoC subunits and to bovine IP 30-kDa, Pd-NQO5, and Ec-NuoB subunits, respectively (Table II). Recently, the PdNQO4 and PdNQO5 subunits were alternatively and individually expressed as soluble proteins in E. coli, showing that neither subunit contains iron-sulfur clusters (47). The same conclusion could be drawn from the sequences of T.thermophilus enzymes. Conserved cysteine residues are indicated by *, # indicates cysteine residues that are predicted to ligate a [2Fe-2S] cluster. An arrow (°) indicates the cysteine residue that is not conserved in the T.thermophilus NQO3 subunit. Studies on the T. thermophilus HB-8 NDH-1

Fig. 3. Comparison of the amino acid sequences of the T. thermophilus NQO3 subunit with its homologues from various organisms. The comparison was conducted using the PILEUP program. Bovine, bovine heart mitochondrial Complex I; P.d., P. denitrificans; T.th., T. thermophilus. The comparison was conducted using the PILEUP program. Bovine, bovine heart mitochondrial Complex I; P.d., P. denitrificans; T.th., T. thermophilus. Conserved cysteine residues are indicated by *, # indicates cysteine residues that are predicted to ligate a [2Fe-2S] cluster. An arrow (°) indicates the cysteine residue that is not conserved in the T. thermophilus NQO3 subunit.
lus subunits. The TthNQO4 subunit contains only one non-conserved cysteine residue (Cys385), whereas the TthNQO5 subunit does not contain cysteine residues. The functional roles of these subunits are not known.

TthNQO6 Subunit—Among the 14 subunits, the TthNQO6 subunit showed the highest sequence identities to its bovine, P. denitrificans, and E. coli equivalents (Fig. 4A and Table II). These subunits are homologous to the HycG subunit of E. coli formate hydrogen lyase. This subunit contains conserved cysteine residues that are also present in TthNQO6 subunit (Cys45, Cys46, Cys111, and Cys140). The cysteine arrangement in the NQO6 subunit and its homologues has been suggested to be analogous to that in the small subunit of nickel-iron hydrogenases (48, 49). In the latter enzymes, involvement of the corresponding cysteines in ligating a [4Fe-4S] cluster has recently been revealed by the three-dimensional crystal structure of Desulfovibrio gigas hydrogenase (50). It remains to be determined whether the NQO6 subunit contains an iron-sulfur cluster.

TthNQO9 Subunit—The TthNQO9 subunit is homologous to the bovine TYK, PdNQ9, and EcNuoI subunits (Fig. 4B and Table II). Sequence suggests that this subunit contains iron-sulfur clusters. The TthNQO9 subunits contains 8 invariant cysteine residues (Cys53...Cys60) that are typical sequence motifs of [4Fe-4S] cluster binding sites as seen in 2,3...[4Fe-4S]ferredoxins (51, 52). Our preliminary results from an expression and reconstitution study of the PdNQ9 subunit have indicated that NQO9 indeed contains two [4Fe-4S] clusters.4

Iron-sulfur cluster N2, which probably plays a key role in the electron transfer pathway as a direct electron donor to quinone, has not yet been assigned to a subunit. The hypothetical cluster in NQO6 and either of those in NQO9 are possible candidates.

TthNQO7, TthNQO8, and TthNQO10–14 Subunits—T. thermophilus NDH-1 gene cluster contains seven genes (NQO7, NQO8, and NQO10–14) that encode subunits homologous to bovine mitochondrial ND3, ND1, ND6, ND4L, ND5, ND4, and

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ND2 gene products and to the other seven subunits of bacterial NDH-1 (Table II). These subunits are extremely hydrophobic and are thought to constitute the integral membrane part of the enzyme complex. The *T. thermophilus* subunits contain a number of hydrophobic stretches that are predicted to span the cytoplasmic membrane (Fig. 5). As has been suggested previously (1, 2, 5, 53, 54), the membrane assembly probably plays an essential role in quinone reduction and proton translocation. The *T. thermophilus* membrane subunits conserve several charged amino acid residues, some of which may be involved in the energy-tranducing mechanism. It is very difficult, however, to discuss specific functional roles of the individual subunits based only on the sequence data. Little is known about the structures of these subunits, and quinone-binding site(s) have not been identified. Thus, much future work is needed before any conclusions can be made.

Expression of the *T. thermophilus* NQO2 (TthNQO2) Subunit in *E. coli*—As mentioned previously, the TthNQO2 subunit contains the four conserved cysteine residues that were shown to ligate a [2Fe-2S] cluster in PdNQO2. The PdNQO2 subunit is one of the well characterized subunits in NDH-1 (26, 42, 55) and has similar physicochemical properties to *Clostridium pasteurianum* binuclear ferredoxin (56). Therefore, to explore the thermostable properties, we attempted to express the TthNQO2 subunit in *E. coli* and purify it. Its physicochemical properties could be directly compared with those of the PdNQO2 subunit. The TthNQO2 subunit was expressed as a soluble protein and could be purified with nickel chelation column chromatography (Fig. 6A). We raised polyclonal antibodies against the expressed TthNQO2 subunit and utilized them to examine the identity of the subunit with the *T. thermophilus* membrane preparation. The antibodies recognized a single polypeptide with an apparent molecular weight of $M_r = 21,000$ (Fig. 6B). These results indicate that the sequences were precisely analyzed and that the subunit was correctly expressed in *E. coli*. The purified TthNQO2 subunit was reddish-brown in color as previously observed with the PdNQO2 subunit. Its absorption spectrum showed great resemblance to that of the PdNQO2 subunit. Characteristic absorption peaks of the iron-sulfur cluster (330, 420, 460, and 550 nm) could be
seen in its oxidized form (Fig. 7A). EPR measurements provided further evidence that the TthNQO2 subunit contains a single [2Fe-2S] cluster with similar properties to the PdNQO2 subunit (Fig. 7B).

**Thermostability of the TthNQO2 Subunit**—It has been reported that the isolated *T. thermophilus* NDH-1 enzyme complex exhibits extremely thermostable properties and is capable of catalyzing NADH oxidation at temperatures over 65°C. In a preliminary experiment, the thermostability of the expressed TthNQO2 subunit was tested by incubating crude preparations (cytoplasmic fraction) at 65°C for various time under anaerobic conditions. The solutions were centrifuged to remove denatured proteins, and the resultant supernatants were subjected to SDS-PAGE. As shown in Fig. 8A, while *E. coli* proteins were readily denatured by the heat treatment, the TthNQO2 subunit remained soluble in the supernatant. These results show that the TthNQO2 subunit is more heat-resistant than *E. coli* proteins. After subsequent purification by nickel chelation column chromatography, the subunit exhibited the characteristic red-brown color, indicating that the [2Fe-2S] cluster also survived the treatment (data not shown). The TthNQO2 subunit was further used in order to assess the degree of thermostability. The purified TthNQO2 subunit (by Protocol 1) was incubated at various temperatures (50–90°C) for 30 min under anaerobic conditions followed by centrifugation at 14,000 rpm for 10 min. Absorption spectra of the supernatants were measured in the visible region. Almost no precipitation was observed up to 65°C, and the absorption spectra of the subunits were unaltered (Fig. 8B, line 1). Notably, no spectral changes occurred at 65°C during incubation up to 3 h (data not shown). When the temperature was elevated to 75–80°C, the subunit started to show some instability such that some portion of the protein was denatured and precipitated (Fig. 8B, lines 2 and 3). At 85°C and above, the denaturing process was further accelerated. The absorption spectrum of the soluble subunits was significantly modified (Fig. 8B, line 4). On the other hand, when the PdNQO2 subunit was heat-treated in the same way, the protein was immediately denatured and precipitated (Fig. 8B, line 5). These results clearly demonstrate that the *T. thermophilus* NDH-1 subunits are extremely stable and thus more suitable to express and study as single proteins than are their mesophilic equivalents. It should be noted that the iron-sulfur cluster in the TthNQO2 subunit is stable only under oxygen-free conditions. In the presence of oxygen, the subunit readily lost its reddish-brown color, indicating that the iron-sulfur cluster is vulnerable to attack by oxygen. This is often seen also in other iron-sulfur proteins (57).

**DISCUSSION**

The present study has revealed that the *T. thermophilus* NDH-1 (eukaryotic Complex I equivalent) genes constitute an operon-like structure that is composed of 14 structural genes (NQO1–14) with no URFs. The *T. thermophilus* NDH-1 is composed of seven predicted transmembrane subunits (NQO7, NQO8, and NQO10–14) and seven peripherally located subunits (NQO1–6 and NQO9), all of which are very homologous to the corresponding subunits of mitochondrial Complex I and other bacterial NDH-1. The order of the 14 structural genes is identical to those of other NDH-1 gene clusters (*P. denitrificans* and *E. coli*) and similar to those encoding other related enzymes, suggesting a phylogenetic relationship between bacterial NDH-1, NAD-reducing hydrogenase, and some related enzymes. These results further substantiate that the 14-subunit structure is the minimal and ubiquitous functional unit of Complex I and NDH-1.

Previous biochemical studies of the isolated *T. thermophilus* NDH-1 have shown that the enzyme contains one FMN and several iron-sulfur clusters as redox components (19). The *T.
Studies on the *T. thermophilus* HB-8 NDH-1

*thermophilus* NDH-1 contains only 40 cysteine residues (while the *P. denitrificans* NDH-1 contains 64 cysteine residues), all of which are located in the extrinsic subunits (Table III). Thirty-two out of 40 cysteine residues are fully conserved among mitochondrial and bacterial Complex I whose amino acid sequences have been reported. As described under “Results,” the *T. thermophilus* NDH-1 has the potential of bearing eight iron-sulfur clusters in common with mitochondrial and bacterial enzyme complexes and probably an additional [2Fe-2S] cluster in the *TthNQO3* subunit, which is unique to *T. thermophilus*, *E. coli*, and *S. typhimurium*. It has been shown by EPR studies that at least three NADH-reducible iron-sulfur clusters (one binuclear and two tetranuclear) and possibly two iron-sulfur clusters (one binuclear and one tetranuclear) with very low redox midpoint potentials are present in the membrane-bound *T. thermophilus* NDH-1 (20). Although exact assignment of these EPR-detectable iron-sulfur clusters remains to be made, these results together with the sequence information in the present study indicate that the *T. thermophilus* NDH-1 possesses at least as many iron-sulfur clusters as its mitochondrial and bacterial equivalents.

However, *T. thermophilus* NDH-1 seems to be unique among the known Site I enzyme complexes from a bioenergetic point of view, because *T. thermophilus* contains only menaquinone-8 (58). Menaquinone-8 has a lower redox midpoint potential than ubiquinone (*E_m,7* = −75 mV for menaquinone/maenaquinol couple; *E_m,7* = +100 mV for ubiquinone/ubiquinol couple). Thus, the free energy available from electron transfer from NADH to menaquinone is much less than when using ubiquinone. Therefore, comparative studies of *T. thermophilus* NDH-1 with ubiquinone-utilizing complexes may provide new insights into Site I energy coupling mechanism.

Thermostability of proteins isolated from not only *T. thermophilus* but also from several other thermophilic microorganisms has recently received considerable attention. Several attempts have been made to clarify the origin of the thermostability, and some principles have been proposed (59–61). Based on the comparative structural studies of thermophilic and mesophilic proteins, it seems likely that structural stability of thermophilic proteins are reinforced by an increment of intrapoly peptide interactions throughout the protein molecule (62–64). The present study has shown that the expressed *TthNQO2* subunit is thermostable, since the subunit and its iron-sulfur cluster survived at 65 °C for 3 h. The same observation has been made for other *T. thermophilus* NDH-1 subunits and subcomplexes expressed in *E. coli*.5 These results indicate that heat resistance resides in individual subunits. The purified *NQO2* subunit and other expressed subunits remained stable at room temperature (22–25 °C) for weeks in an anaerobic chamber without losing the iron-sulfur cluster. The availability of these stable subunits give us the possibility to

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### Table III

| Subunits | No. of cysteine residues | Conserved cysteine residues | Iron-sulfur clusters |
|----------|--------------------------|-----------------------------|----------------------|
| NQO1     | 6 (4)                    | Cys182, Cys183, Cys186, Cys189, Cys190 | [4Fe-4S] (N3) |
| NQO2     | 6 (4)                    | Cys183, Cys186, Cys189, Cys190 | [2Fe-2S] (N1a) |
| NQO3     | 15 (11)                  | Cys24, Cys65, Cys66, Cys67, Cys110, Cys112, Cys124, Cys128, Cys134, Cys140, Cys144, Cys145, Cys146, Cys150, Cys206, Cys209, Cys210 | [4Fe-4S] (N4) |
| NQO6     | 4 (4)                    | Cys65, Cys66, Cys111, Cys140 | [2Fe-2S] (N1a) |
| NQO9     | 8 (8)                    | Cys53, Cys56, Cys69, Cys93, Cys98, Cys101, Cys104, Cys108 | [4Fe-4S] (N27) |
| **Total**| **40** (32)              |                             | 8–9 iron-sulfur clusters |

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5 T. Yano and T. Yagi, unpublished data.
investigate subunit-subunit interaction by reconstituting the entire enzyme complex from individual components as demonstrated for ATPase (65). Furthermore, significant stability is also a great advantage for structural studies of the complex using x-ray crystallography or NMR spectroscopy.

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