FEBS Letters 356 (1994) 333–338

FEBS 14888

Cloning and sequencing of four structural genes for the Na^+-translocating NADH–ubiquinone oxidoreductase of *Vibrio alginolyticus*

P. Beattie[a], K. Tan[a], R.M. Bourne[b], D. Leach[a], P.R. Rich[b], F.B. Ward[a,*]

[a]*Institute for Cell and Molecular Biology, Darwin Building, Edinburgh University, Mayfield Rd, Edinburgh EH9 3JR, UK*
[b]*Glynn Research Institute, Bodmin, PL30 4AU, UK*

Received 26 October 1994

Abstract Oligonucleotide probes based on the N-terminal amino acid sequences of the NqrA and NqrC subunits were used to clone genes for the Na^+-dependent NADH–ubiquinone oxidoreductase complex from *Vibrio alginolyticus*. Four consecutive ORFs were identified encoding subunit proteins of 48.6, 46.8, 27.7 and 22.6 kDa, respectively (NqrA–D). A further ORF, showing 71% homology to the BolA protein of *Escherichia coli*, was located upstream. From sequence comparisons, we conclude that the Na^+-dependent NADH–ubiquinone oxidoreductase complex of *V. alginolyticus* is clearly distinct from the corresponding H^+-dependent enzymes of both prokaryotes and eukaryotes.

Key words: Cloning; Nucleotide sequence; NADH–ubiquinone oxidoreductase; *Vibrio alginolyticus*

1. Introduction

NADH–ubiquinone oxidoreductase (complex I) transfers electrons from NADH to ubiquinone and links this to proton translocation across the mitochondrial inner membrane. Complex I from eukaryotes has been extensively studied in *Bos taurus* and *Neurospora crassa*; the *B. taurus* complex I is thought to comprise 41 subunits and can be biochemically split into three fractions: FP (flavoprotein), IP (iron-sulphur protein) and HP (hydrophobic protein-ND subunits) [1]. The FP fraction contains a catalytic 51 kDa subunit containing NADH- and FMN-binding sites, a 24 kDa [2Fe-2S] subunit and a 10 kDa subunit. Seven of the genes are mitochondrially encoded and are highly hydrophobic; one subunit, ND1, contains a ubiquinone-binding site while another, ND2, is reactive towards both rotenone and DCCD [1]. Recently, gene clusters coding for proton-translocating NADH–ubiquinone oxidoreductases have been identified in two prokaryotes: *Paracoccus denitrificans* and *Escherichia coli* [2,3].

By contrast, the marine bacterium *Vibrio alginolyticus* has been shown to produce an electrochemical Na^+ gradient in aerobic respiration as the result of a sodium-translocating NADH–ubiquinone oxidoreductase, which is induced at alkaline pH [4–7]. The resulting sodium motive force is used to drive ATP synthesis, flagellar rotation and solute transport [1,8,9]. This alternative to H^+ coupling in membrane reactions enables bacteria to maintain a cytoplasmic pH near to neutrality in an alkaline environment. The Na^+-dependent NADH–ubiquinone oxidoreductase complex is distinct from a Na^+-independent NADH–ubiquinone oxidoreductase also found in *V. alginolyticus*, in that Na^+-dependent NADH–ubiquinone oxidoreductase is sensitive to inhibition by silver ions and by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), can generate a membrane potential, reduces quinone using a one-electron pathway (rather than a two-electron pathway), and can utilize the NADH analogue deaminoNADH[10–13]. Early biochemical studies indicated that the purified Na^+-dependent NADH–ubiquinone oxidoreductase complex is composed of three subunits: α, β and γ, with apparent M_r of 52, 46 and 32 kDa, respectively [14]. The FAD-binding β-subunit demonstrates NADH dehydrogenase activity, accepting electrons from NADH and reducing hydrophilic quinones, such as menadione, by a one-electron transfer reaction to produce semiquinones. This reaction is independent of Na^+. However, the reduction of hydrophobic quinones, such as ubiquinone, is Na^+-dependent and is catalyzed by the FMN-containing α-subunit in the presence of the β-subunit [10,14,15]. The γ subunit was proposed to increase the affinity of the β-subunit for quinones and to assist in the electron transfer reaction from β to α [16]. As part of a programme to study the structure and function of the sodium pump complex in *V. alginolyticus*, we have cloned and sequenced four genes of the Na^+-dependent NADH-ubiquinone oxidoreductase complex and the results are presented in this communication.

2. Materials and methods

2.1. Protein purification and N-terminal sequencing

Cells of *V. alginolyticus* were grown at 30°C in a medium of 5 g/l peptone, 5 g/l yeast extract, 39 g/l NaCl and 5 g/l dipotassium hydrogen orthophosphate. The initial pH of the medium was adjusted to 8.5 with Tris base. The culture was aerated with 95% O_2/5% CO_2 and pH was maintained above 8.2 with NaOH. Cells were harvested in late-log phase and washed in 20 mM Tris-HCl, 1 M NaCl, pH 8.0. Cells were lysed by osmotic shock in 40 mM Tris-HCl, 5 mM EDTA at pH 8.0, treated with RNase and DNase, pelleted by centrifugation at $18{,}000 \times g_{av}$ and stored frozen in 20 mM Tris-HCl, 10 mM NaCl, 10% (v/v) glycerol, pH 8.0.

Membranes were prewashed with 0.1% Synperonic PE/F68 detergent and the Na^+-dependent NADH–ubiquinone oxidoreductase solubilized with 1% (w/v) lauryl maltoside in 20 mM Tris-HCl, 10 mM NaCl, 5% (v/v) glycerol, pH 8.0. Insoluble material was removed by centrifugation at $70{,}000 \times g_{av}$ for 60 min. The supernatant was applied to a column of DEAE Sepharose CL6B and eluted in 0.5% (w/v) lauryl maltoside, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, and then further purified by preparative gel electrophoresis (BioRad 491 Prep Cell) using a 4% (w/v) acrylamide gel containing 1% (w/v) agarose in a gel buffer

*Corresponding author. Fax: (44) (31) 668 3870.
E-mail: bward@srv0.bio.ed.ac.uk

Abbreviations: ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; complex I, H^+-translocating NADH–ubiquinone oxidoreductase complex.

The sequence has the EMBL nucleotide sequence database accession number Z37111.

SSDI 0014-5793(94)01275-X

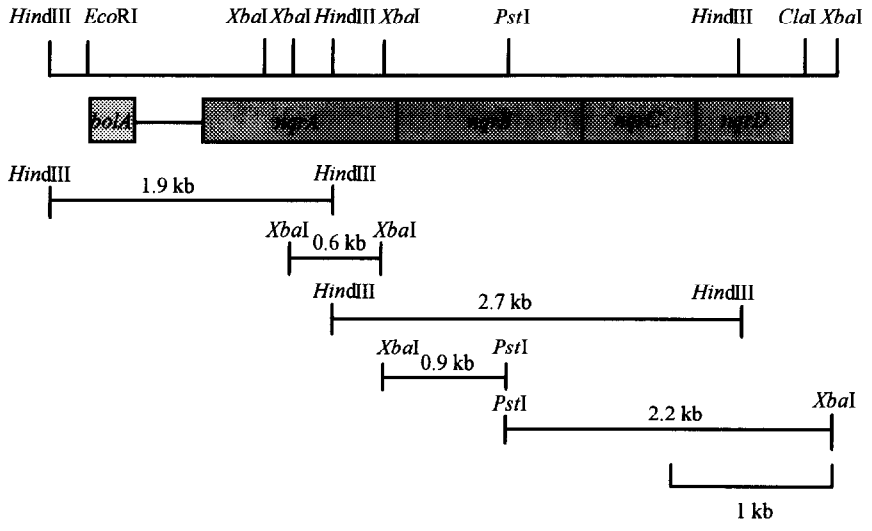


Fig. 1. Restriction and gene map of the upstream cluster of *nqr* genes in *Vibrio alginolyticus*.

of 0.1% (w/v) lauryl maltoside, 1 M Tris-HCl, pH 8.8. The elution buffer was 0.1% (w/v) lauryl maltoside, 20 mM Tris-HCl, 10 mM NaCl, 5% (v/v) glycerol, pH 8.0. Individual polypeptides were separated on 10% SDS-PAGE gels using appropriate molecular weight markers and electroblotted onto PVDF membranes using the method of Matsudaira [17]. Proteins were stained with Amido black and the excised bands analyzed for N-terminal sequence on an Applied Biosystems Analyser (WELMET). Activity of NADH dehydrogenase was detected on native gels using deaminoNADH and Nitrotetrazolium blue.

2.2. Construction and screening of a genomic DNA library

Genomic DNA was purified from *V. alginolyticus* NCIMB 11038 by standard methods, restricted with *Eco*RI and used to construct a library in the λ vector NM1149 [18]. Oligonucleotide probes were made by OSWEL (University of Edinburgh). These were probe 1, 5′-GATG-AT(CT)AC(ACGT)AT(CT)AA(AG)AA(AG)GG-3′ (degeneracy 64, based on MITIKKG of the α-subunit); probe 2, 5′-CA(AG)AA (AG)GA(AG)GA(AG)AC(AGCT)AA-3′ (degeneracy 32, based on QKEETK of the γ1-subunit); probe 3, 5′-CC(AGT)CA(AG)GC-(AGT)GA(AG)CA(AG)GT-3′ (degeneracy 72, based on PQAEQV of the γ1-subunit); probe 4, 5′-AA(CT)AA(CT)GA(CT)TC(AGT)AT (ACT)GG-3′ (degeneracy 72, based on NNDSIG of the γ2-subunit); and probe 5, 5′-AA(CT)AA(CT)GA(CT)AG(CT)AT(ACT)GG-3′ (degeneracy 48, based on NNDSIG of the γ2-subunit). These were end-labelled with ^{32}P and used for plaque hybridization at low stringency (2 × SSC, 22°C). Positively hybridizing plaques were picked, purified twice and re-probed at higher stringency (0.1 × SSC, 30°C).

2.3. Subcloning and sequencing

DNA was prepared from positively hybridizing clones, cut with the appropriate restriction enzyme and the fragments sized by agarose gel-electrophoresis. The DNA was blotted onto nitrocellulose filters, and Southern hybridization with the ^{32}P-labelled probes was used to identify the 5′ end of the corresponding *nqr* gene. Nucleotide sequence analysis was carried out on the double-stranded templates using a T7 polymerase method (Pharmacia). For GC-rich regions, a method using *Taq* polymerase and deazaGTP (Promega) was preferred. Both strands were sequenced using either vector primers (Universal and Reverse) or synthesized oligonucleotide primers (OSWEL). Sequences were analyzed using both the University of Wisconsin Genetics Computer Group programs and BLITZ (EMBL, Heidelberg) [19].

3. Results

3.1. N-terminal sequencing of the α- and γ-subunits

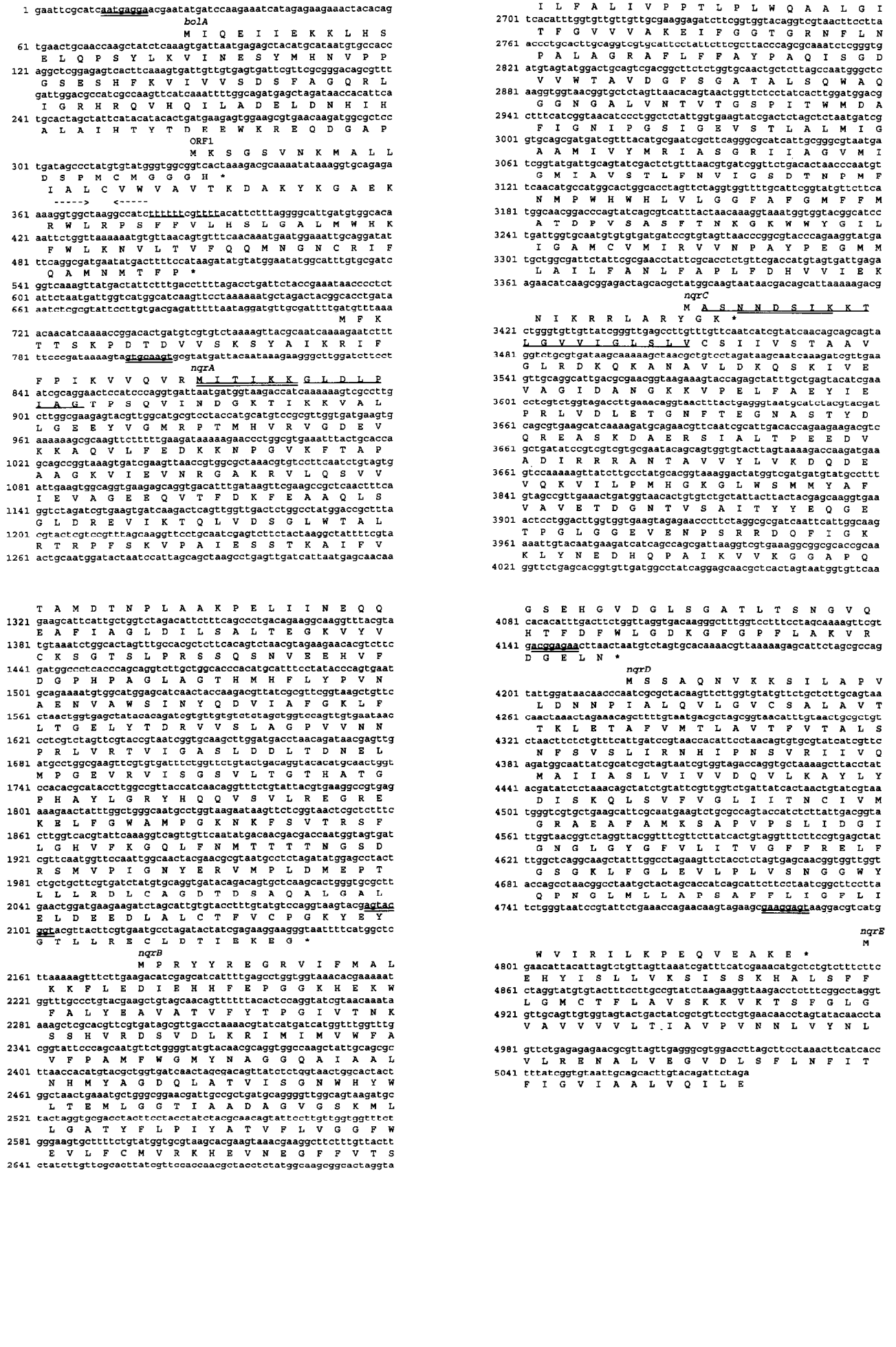
The Na^+-dependent NADH–ubiquinone oxidoreductase complex from *V. alginolyticus* was partially purified as described above. Zymogram staining of enzyme activity on non-denaturing PAGE gels indicated a M_r of 254 kDa for the complex. The subunits were separated on SDS-PAGE gels. From the partially purified preparations of NADH–ubiquinone reductase, we were able to identify four subunits: α (55 kDa), β (50 kDa), γ1 (33 kDa) and γ2 (30 kDa). These were blotted onto PVDF membranes and the N-terminal sequences determined as MITIKKGLDL for the α-subunit, QKEETKTEA-APQAEQVQ for the γ1-subunit, and ASNNDSIKKTLGVV-IGL-LV for the γ2-subunit. No N-terminal sequence for the β subunit could be obtained because of blocking at the N-terminus.

3.2. Identification and sequencing of nqrA *and* nqrC

From the N-terminal amino acid sequences, oligonucleotide probes were designed to probe the λ library. Several clones hybridizing to probe 1 (*nqrA*, α-subunit) were isolated and purified. From one positive clone, a 10.7 kb *Eco*RI insert was mapped and shown to give five *Hin*dIII fragments of approximate sizes 2.9, 2.7, 1.9, 1.65 and 1.0 kb. A partial restriction map of the cloned DNA is shown in Fig. 1. The 1.9 kb *Hin*dIII fragment hybridized with probe 1 and was subcloned into the sequencing vector pTZ19R (Pharmacia). DNA sequencing showed that the first 254 bp of DNA were of λ origin, representing the large region between *Hin*dIII and *Eco*RI in this vector and thereby locating the 1.9 kb fragment as being on the left end of the original 10.7 kb *Eco*RI fragment. Within the 1.9 kb *Hin*dIII fragment, three ORFs were detected. The first encodes a polypeptide of 102 amino acid residues, which showed

→

Fig. 2. The nucleotide and deduced protein sequences of the *nqr* and *bolA* genes. Double underlined protein sequence indicates sequence used to design oligonucleotide probes. Singly and doubly underlined protein sequence denotes that determined by amino acid sequencing. Double underlined nucleotide sequence denotes putative Shine–Dalgarno sequences. The region of nucleotide sequence with opposing arrows above the sequence represents a region of dyad symmetry adjacent to a poly(dT) region (single underline) that could act as a transcription terminator for the *bolA* gene.


bolA
ORF1
nqrA
nqrB
nqrC
nqrD
nqrE

71% similarity and 55% identity with the *bolA* gene of *E. coli* [20]. The second ORF encodes a putative polypeptide of 80 amino acid residues. No strong homologies could be found to other proteins in the SWISSPROT database, but a 24% identity over 33 residues with chain 5 of the putative chloroplast NADH–plastoquinone oxidoreductase from *Oryza sativa* was observed [21]. The third ORF contained the sequence MITIKKG determined from the α-subunit and extended to the end of the 1.9 kb *Hin*dIII fragment. Further sequencing of the adjacent 2.7 kb *Hin*dIII fragment, the overlapping 0.6 kb *Xba*I and the 0.9 kb *Xba*I–*Pst*I fragments (Fig. 1) gave the sequence of the C-terminal portion (Fig. 2). Sequence comparison using the MOTIFS program detected no evidence of the presence of the highly conserved GAG(A/R)Y motif found in the FMN-containing subunit of complex I [1–3].

Hybridization experiments with probe 5 to detect the gene for the γ2-subunit showed that this gene was located on a 3.1 kb *Xba*I fragment subcloned from the original 10.7 kb *Eco*RI clone. This fragment contained a unique *Pst*I site that divided the fragment into a 2.2 kb and an 0.9 kb fragment (Fig. 1). Probe 5 reacted with the 2.2 kb fragment. Thus, the *nqrC* gene was shown to map to the same region of the chromosome as the *nqrA* gene and comprised an ORF of 768 bp in the same orientation as the *nqrA* gene and 1.2 kb downstream from the end of the *nqrA* gene. The N-terminal amino acid from sequencing agreed exactly with that obtained by protein analysis.

3.3. *Sequencing of* nqrB *and* nqrD

Two further ORFs, corresponding to *nqrB* and *nqrD*, were found on further sequencing of the region between *nqrA* and *nqrC* and the region immediately downstream of *nqrC*. *NqrB* is located immediately downstream of *nqrA* and extends 1278 bp from the 0.9 kb *Xba*I–*Pst*I fragment into the 2.2 kb *Pst*I–*Xba*I fragment. Downstream of *nqrC*, an ORF of 630 bp was found and this was termed *nqrD*. We have also found an ORF, designated *nqrE*, adjacent to *nqrD*, which has not been completely sequenced. Hydropathy plots of NqrB and NqrD indicate that they are integral membrane proteins that possess a number of putative membrane-spanning helices (data not shown).

Sequence comparisons of the proteins encoded by the *nqrA–D* genes was carried out using the BLITZ programme, over a range of PAM [22] values (40–350), to determine homology with other proteins in the SWISSPROT database over short and long regions. For NqrB and NqrD, a low homology to hydrophobic subunits of complex I from both eukaryotes and prokaryotes was apparent at high PAM values (250–350). However, these homologies were not to a single subunit of Complex I but to a number of different hydrophobic subunits. For example NqrB showed homology to chain 4 (equivalent to ND4) of *Paramecium tetraurelia*, chains 1 (equivalent to ND1) and 5 (equivalent to ND5) of *Oenothera bertiana* and NqrD to chain 4 of *Synechocystis* spp., subunits 13 and 14 (equivalent to bovine ND4 and ND2, respectively) of *P. denitrificans*, and chain 5 of both *Anopheles gambiae* and *Drosophila yakuba*. At PAM 250 the matches for NqrB and NqrD were 8 and 14% respectively; the corresponding values at PAM 350 were 15 and 21%. By contrast the same comparisons between the corresponding subunits of complex I from various species gave values in the range 20–>90%. Comparison of the predicted number, the number of results expected by chance to have a score greater or equal to the given score for the specific comparison, showed very low values for the corresponding hydrophobic subunits from different species (i.e. statistically meaningful) and also showed the previously described relationships between chain 5, chain 4 and chain 2 of complex I from different species [23]. The predicted numbers for comparisons between NqrD and hydrophobic subunits of complex I were high and of the same order as the predicted numbers for comparisons between chains 4 and 6, or between chains 4 and 1 of different species. In addition NqrB and NqrD also showed low homology to ion transporters over a range of PAM values (40–350). NqrD showed homology to Na^+-channel proteins over individual transmembrane segments (e.g. CIN2 of *Rattus norvegicus*). The Na^+-channel proteins have repeated units consisting of three transmembrane helices (S1–S3) followed by a positively charged segment (S4), thought to be the voltage sensor, and two further transmembrane helices [24,25]. By contrast NqrA and NqrC showed no obvious homology to proteins in the database. The deduced properties of the four completely sequenced subunits are shown in Table 1.

4. Discussion

Independent evidence shows that the structural genes for the α- (*nqrA*) and γ2- (*nqrC*) subunits are located close together on the chromosome of *V. alginolyticus*. The amino acid residues corresponding to the nucleotide sequences of the oligonucleotide probes for both *nqrA* and *nqrC* were found at the N-termini of the predicted amino sequences, and the amino acid sequence determined from nucleotide sequencing agreed well with that obtained by protein sequencing for 19 residues of NqrC and 10

Table 1
Summary of properties of polypeptides encoded by the *nqrA–D* genes of *V. alginolyticus*

Subunit	NqrA	NqrB	NqrC	NqrD
M_r	48,622	46,809	27,672	22,602
No. residues	446	426	257	210
pI	5.58	9.12	4.91	9.28
N-Terminus	MITIKKGLDL-	MPRYYREGV-	MASNNDSIKK-	MSSAQNVKKS-
Predicted no. of membrane-spanning helices	0	6–12	1 (N-terminal)	4–6
Comments		Very hydrophobic Weak homologies with complex I subunits and sodium channel proteins	Hydrophobic N-terminal region	Very hydrophobic Weak homologies with complex I subunits and sodium transporters

residues for NqrA (Fig. 1), confirming that the sequences of these two genes do correspond to the α- and γ2-subunits of the partially purified Na^+-dependent NADH–ubiquinone reductase. The serine codon of AGC found for residue 7 of *nqrC* is consistent with the observation that probe 5, but not probe 4, hybridized to the N-terminal region of the *nqrC* gene. The predicted M_r values of 48.6 kDa for the α-subunit and 27.6 kDa for the γ2-subunit are in reasonable agreement with the experimental values of 55 and 30 kDa determined from SDS-PAGE gels, as it is well documented that membrane proteins give poorer correlation than soluble proteins. Although no FMN-binding site was detected by sequence analysis, this does not preclude the α-subunit having FMN as a prosthetic group. Searching the databases indicates that even for the highly conserved fumarate reductases, the FAD consensus sequence RSH[ST]x(2)-A-x-GG is only found in a limited number of proteins. To date, a sequence coding for the catalytic subunit of the enzyme and containing NADH- and flavin-binding sites has not been identified and this gene may be located further downstream. Searching for binding sites using the program MOTIFS did not reveal any NADH-, [Fe-S]-, or FAD-binding sites in the four genes. For *E. coli*, *P. denitrificans* and *B. taurus* there is strong conservation of these binding sites in the c.51 kDa catalytic subunit [2,3].

Comparison of the amino acid sequences for proteins NqrA–D indicated that they are not closely related to the functionally corresponding complex I of either prokaryotes or eukaryotes. Indeed the observation that the low homologies between NqrD and subunits ND1, ND2, ND4 and ND5 of complex I from various species are very similar support the postulate that the Na^+-dependent NADH–ubiquinone oxidoreductase are either unrelated or diverged early in evolution from complex I. It is pertinent to note that both ND2, ND4 and ND5 subunits have been postulated to evolve from a common ancestor by gene duplication [26]. A further distinction between the Na^+-dependent NADH–ubiquinone oxidoreductase and complex I lies in the operon structure. For *E. coli* and *P. denitrificans* the gene order is identical. For *E. coli* the subunit sizes in kDa and the gene order are (*B. taurus* equivalents in brackets): 16 (ND3), 25 (20 IP), 22 (23 IP), 46 (49 IP), 19 (24 FP), 50 (51 FP), 75 (IP) followed by the mainly very hydrophobic proteins 36 (ND1), 20 (23), 20 (ND6), 11 (ND4L), 66 (ND5), 51 (ND4) and 52 (ND2) [2]. The genes for the peripheral membrane proteins are adjacent (*nuoC–G*). For *V. alginolyticus* the subunit sizes in kDa are, in order: 49, 47 (HP), 28 and 23 (HP), which bears little correspondence to the arrangement for *E. coli*. The divergence between the Na^+-dependent NADH–ubiquinone oxidoreductase of *V. alginolyticus* and complex I of *E. coli* is in contrast to the case for other genes, eg. *bolA* and *unc* genes of *V. alginolyticus* and *E. coli*, which are much more closely related [27]. The extent and nature of the AT-rich promoter control region for *nqrA* is as yet uncertain. Downstream of *bolA* is a region of dyad symmetry followed by a T-rich region (Fig. 2) that is characteristic of a rho-independent transcriptional regulator [28]. It is preceded by an A-rich sequence upstream of the GC-rich motif that is complementary to the T-rich tail. Such A-rich sequences are thought to increase the efficiency of termination [29]. The location of the *bolA* terminator region within ORF1 makes it likely that this putative gene product is not produced as a translational product. Evidence from N-terminal sequencing strongly indicates that the NqrA subunit begins MITIKK etc., rather than MFKTTS, but further experiments to determine the transcriptional and translational starts are in progress.

In summary we have cloned and sequenced four genes for the Na^+-dependent NADH–ubiquinone oxidoreductase of *Vibrio alginolyticus*. Our data is consistent with the hypotheses that Na^+-dependent NADH–ubiquinone oxidoreductase is distinct from the H^+-translocating complex I, that the *nqr* genes form part of an operon, that the complex is larger than the three subunits isolated previously, and that at least two proteins (NqrB and NqrD) may form ion channels. Further experiments are in progress to determine the function of these subunits and the regulation of *nqr* gene expression.

Acknowledgements: Financial support from Glynn Research Foundation is gratefully acknowledged. We thank Professor R. Ambler and Dr. D. Finnegan in Edinburgh for advice and support and Dr. B. Meunier at Glynn for advice.

References

[1] Walker, J.E. (1992) Quart. Rev. Biophys. 25, 253–324.
[2] Weidner, U., Geier, G., Ptock, A., Friedrich, T., Leif, H. and Weiss, H. (1993) J. Mol. Biol. 233, 109–122.
[3] Xu, X., Matsuno-Yagi, A. and Yagi, T. (1993) Biochemistry 32, 968–981.
[4] Skulachev, V.P. (1988) Membrane Bioenergetics, pp. 293–326, Springer-Verlag, Berlin.
[5] Tokuda, H. and Unemoto, T. (1981) Biochem. Biophys. Res. Commun. 102, 265–271.
[6] Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014.
[7] Unemoto, T., Tokuda, H. and Hayashi, M. (1990) in: Bacterial Energetics (Krulwiche, T.A., Ed.) pp. 35–54, Academic Press, San Diego.
[8] Avetisyan, A.V., Bogachev, A.V., Murtasina, R.A. and Skulachev, V.P. (1993) FEBS Lett. 317, 267–270.
[9] Dimroth, P. (1987) Microbiol. Rev. 51, 320–340.
[10] Unemoto, T. and Hayashi, M. (1989) J. Bioenerg. Biomembr. 21, 649–662.
[11] Hayashi, M., Miyoshi, T., Sato, M. and Unemoto, T. (1992) Biochim. Biophys. Acta 1099, 145–151.
[12] Unemoto, T., Miyoshi, T. and Hayashi, M. (1992) FEBS Lett. 306, 51–53.
[13] Unemoto, T., Ogura, T. and Hayashi, M. (1993) Biochim. Biophys. Acta 1183, 201–205.
[14] Hayashi, M. and Unemoto, T. (1986) FEBS Lett. 202, 327–330.
[15] Hayashi, M. and Unemoto, T. (1987b) Biochim. Biophys. Acta 890, 47–54.
[16] Hayashi, M. and Unemoto, T. (1987a) Biochim. Biophys. Acta 767, 470–478.
[??] Tokuda, H. and Kogure, K. (1989) J. Gen. Microbiol. 135, 703–709.
[17] Matsudaira, P.T. (1989) A Practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press.
[18] Murray, N.E. (1983) in: Lambda II (Hendrix, R.W., Roberts, J.W., Stahl, F.W., Weisberg, R.A., Eds.) pp. 395–432, Cold Spring Harbor Laboratory.
[19] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[20] Aldea, M., Garrido, T., Hernandez-Chico, C., Vicente, M. and Kushner, S.R. (1989) EMBO J. 8, 3923–3931.
[21] Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.-R., Meng, B.-Y., Li, Y.-Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K. and Sugiura, M. (1989) Mol. Gen. Genet. 217, 185–194.
[22] Schwarz, R.M. and Dayhoff, M.O. (1978) in: Atlas of Protein Structure and Function (Dayhoff, M.O., Ed.) pp. 353–358, National Biomedical Research Foundation.

[23] Fearnley, I.M. and Walker, J.E. (1992) Biochim. Biophys. Acta 1140, 105–134.
[24] Noda, M., Okeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. and Numa, S. (1986) Nature 320, 188–192.
[25] Catterall, W.A. (1988) Annu. Rev. Biochem. 55, 953–985.
[26] Kikuno, R. and Miyata, T. (1985) FEBS Lett. 189, 85–88.
[27] Krumholz, L.R., Esser, U. and Simoni, R.D. (1989) Nucleic Acids Res. 16, 7993–7994.
[28] d'Aubenton Carafa, Y., Brody, E. and Thermes, C. (1990) J. Mol. Biol. 216, 835–858.
[29] Wright, J.J., Kumar, A. and Hayward, R.S. (1992) EMBO J. 11, 1957–1964.