Expression and mutagenesis of the NqrC subunit of the NQR respiratory Na^+ pump from *Vibrio cholerae* with covalently attached FMN

Blanca Barquera[a,*], Claudia C. Häse[b], Robert B. Gennis[a]

[a] *Department of Biochemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Street, Urbana, IL 61801, USA*
[b] *Department of Infectious Diseases, St. Jude Children's Research Hospital, 332 North Lauderdale Street, Memphis, TN 38105-2794, USA*

Received 15 January 2001; accepted 26 January 2001

First published online 19 February 2001

Edited by Matti Saraste

Abstract The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) is present in the membranes of a number of marine bacteria and pathogenic bacteria. Two of the six subunits of the Na^+-NQR, NqrB and NqrC, have been previously shown to contain covalently bound flavin adenine mononucleotide (FMN). In the current work, the cloning of *nqrC* from *Vibrio cholerae* is reported. The gene has been expressed in *V. cholerae* and shown to contain one equivalent of covalently bound FMN. In contrast, no covalent flavin was detected when threonine-225 was replaced by leucine. The data show that the FMN attachment does not require assembly of the enzyme and are consistent with the unusual threonine attachment site.

Key words: Sodium; NADH; Ubiquinone; Electron transport; Flavin; Site-directed mutagenesis; *Vibrio cholerae*

1. Introduction

The Na^+-translocating NADH:ubiquinone oxidoreductase (Na^+-NQR) is a unique redox-driven sodium pump which functions as an entry point for electrons into the respiratory chains of a number of marine and pathogenic bacteria [1–6]. The enzyme accepts reducing equivalents from NADH and donates them to the quinone pool [1–5]. The energy from this redox reaction is used to pump sodium ions from the inner to the outer side of the cell membrane, creating a sodium motive force which powers the flagellum and drives other metabolic processes [7–9]. The *nqr* operon consists of six genes, which have been cloned from *Vibrio alginolyticus* and *Vibrio harveyi* [4,10,11]. The corresponding Na^+-NQR enzyme complexes have been purified and characterized, showing that the six predicted subunits are all present. The enzyme contains several redox active cofactors which are presumably involved in electron transfer through the enzyme. One of the subunits, NqrF, contains binding motifs for the substrate, NADH, and two prosthetic groups, flavin adenine dinucleotide and a 2Fe–2S center. These redox centers are likely to be the immediate acceptors of electrons from NADH [6,12,13]. NqrB and C have each been shown to contain covalently bound flavin adenine mononucleotide (FMN) [4,14,15].

In a previous report [4], we provided the first direct evidence that NqrC (from the *V. harveyi* enzyme) contains covalently bound flavin, most likely FMN. Histidine-219 (*V. harveyi* numbering) was identified as the possible attachment site for the flavin. Subsequently, Unemoto and colleagues [14,15] reported that the *V. alginolyticus* enzyme contains FMN covalently bound to NqrB in addition to the flavin attached to NqrC. The attachment site for the FMN was identified as threonine in each subunit by protein sequencing (T223 for the *V. alginolyticus* NqrC subunit). Sequence alignment (not shown) of the NqrC subunits from a variety of prokaryotes shows that the threonine attachment site is almost fully conserved, but is replaced by serine in *Neisseria meningitidis*. The nearby histidine is conserved in all but *Chlamydia* and *Porphyromonas gingivalis*.

It has recently been shown that *Vibrio cholerae* also contains Na^+-NQR and that this respiratory sodium pump plays a role in toxin expression [16]. In the present work, the *nqrC* gene from *V. cholerae* has been cloned and expressed in *V. cholerae*. The histidine-tagged NqrC membrane protein was isolated and shown to contain one equivalent of covalently attached FMN. Expression of *nqrC* in *Escherichia coli* results in polypeptide without covalent flavin. Site-directed mutagenesis of the predicted threonine attachment site (T225L) eliminates flavin binding by NqrC. The histidine corresponding to H219 in the *V. harveyi* subunit was also replaced by a leucine and shown not to be essential for covalent flavin attachment. The results show that T225 is the likely site of attachment of FMN to the *V. cholerae* NqrC subunit, and that assembly of the intact six-subunit enzyme complex is not necessary for flavin attachment.

2. Materials and methods

2.1. Bacterial strains, growth conditions and genetic manipulation

E. coli TOP10 (Invitrogen) and XL-1 (Stratagene) were used for cloning. *V. cholerae*, strain O395-N1, was used as host for the expression of *nqr*C-pBAD (wild-type and mutants) and for *nqrB*-pBAD. Strains were grown in LB medium at 37°C. When necessary, ampicillin (Amp) and streptomycin (Str) were added to final concentrations of 100 μg/ml and 50 μg/ml, respectively.

2.1.1. Cloning and expression of nqrC and nqrB. The *nqrC* gene was expressed using the pBAD system (Invitrogen). The vector also contains a 6×-His tag at the C-terminal which allowed the protein to be purified in a single chromatographic step. A 765 bp DNA fragment including the complete *nqrC* gene, except the termination codon, was prepared by PCR and then cloned into the pBAD-TOPO vector. *V. cholerae* (strain O395-N1) genomic DNA was used as a template for

*Corresponding author.
E-mail: barquera@scs.uiuc.edu

Abbreviations: FMN, flavin adenine mononucleotide; NTA, nitrilotriacetic acid; LDAO, lauryldimethylamine-*N*-oxide; SDS, sodium dodecyl sulfate

0014-5793/01/$20.00
PII: S0014-5793(01)02224-4

this reaction. The genomic DNA was prepared using the Wizard genomic DNA kit from Promega. PCR reaction was carried out using Vent polymerase (New England Biolabs). At the end of the extension an additional incubation of the PCR product with Taq polymerase (Promega) and deoxy adenosine triphosphate was performed to add a hanging A to the PCR product. The construct was used to transform TOP10 competent cells and plated on LB-agar-Amp. Plasmid DNA was prepared using a Wizard miniprep kit (Promega). The DNA obtained from the transformants was checked by restriction digestion and by DNA sequencing at the University of Illinois Biotechnology Center. *nqrB* was cloned using the same procedure. A 1.2 kb PCR fragment was cloned into the pBAD-TOPO vector and XL-1 competent cells were transformed. The DNA of the cloned subunits was completely sequenced and no errors were detected.

The constructed plasmids were then introduced into *V. cholerae* by electroporation. Competent cells were prepared using a 50 ml culture in the log phase. Cells were harvested and washed twice with 1 mM $CaCl_2$ solution and then resuspended in 1 mM $CaCl_2$, 10% glycerol. Electroporation was performed in a Bio-Rad 'Gene Pulser' (25 μF, 2 kV, 200 Ω, 4 ms time constant). The electroporated cells were plated on LB-Amp-Str plates and incubated at 37°C.

2.2. Expression and protein purification

The expression of *nqrC* and *nqrB* was checked at different arabinose concentrations. Cells were grown to middle log phase (60 Klett units), arabinose was added, and then the growth continued until end of the log phase. Cells were grown in 2 l flasks (1 l of LB medium per flask; 24 l total) in the presence of Amp and Str. Arabinose (0.2%) was added during the early log phase and growth continued until the cells reached the end of log phase. Cells were harvested and washed with 10 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM $MgSO_4$, 4-(2-aminoethyl)-benzenesulfonyl fluoride and deoxyribonuclease. The washed cells were broken by passing them through a 'Microfluidizer' (Watts Fluidair, Inc.) three times. The suspension was centrifuged at $13\,680\times g$ to remove cell debris and the supernatant was centrifuged at $130\,000\times g$ to pellet the membranes.

The membrane fraction was washed with 50 mM Na-phosphate, pH 8 containing 300 mM NaCl and 5% glycerol. Membranes were frozen at −80°C until needed.

Protein purification. Recombinant NqrC and the mutant derivatives were purified using a Ni-nitrilotriacetic acid (NTA) (Qiagen) column. Approximately 1 mg of recombinant protein was used per ml of resin. Membranes were solubilized in Na-phosphate buffer containing 1% lauryldimethylamine-*N*-oxide (LDAO), 5 mM imidazole and then incubated at 4°C for 30 min. The solubilization mix was centrifuged at $130\,000\times g$ for 30 min. Then the supernatant was mixed with Ni-NTA resin equilibrated with 50 mM Na-phosphate pH 8, containing 300 mM NaCl, 5% glycerol, 5 mM imidazole, 0.1% LDAO (buffer 1) and incubated with mild agitation for 1 h at 4°C. The mixture was loaded into a column and washed with five volumes of buffer 1 followed by five volumes of buffer 1 containing 10 mM imidazole. Then the protein was eluted from the column using buffer 1 containing 100 mM imidazole. The fractions containing NqrC were collected and passed through a gel filtration column (PD-10 column, Sephadex G-25 from Pharmacia) to remove imidazole. The protein was washed from the column using the Na-phosphate buffer, pH 8, with 300 mM NaCl, containing 0.3% LDAO and 10 mM EDTA. The purified protein, which has a yellow color, was concentrated and immediately frozen in liquid N_2 and stored at −80°C.

2.3. Site-directed mutagenesis

Mutants were made with the 'Quick Change' method from Stratagene, using the *nqrC*-pBAD construct as template, and were verified by DNA sequencing.

2.4. Visible and fluorescence spectroscopy

Air oxidized-minus-reduced visible spectra of the purified proteins were recorded on a Shimadzu UV-2101PC spectrophotometer using Na-phosphate buffer, pH 8, containing 10 mM EDTA, 300 mM NaCl, 5% glycerol and 0.3% LDAO, and sodium dithionite as reductant.

For analysis of the covalent flavin, the purified proteins were first precipitated using trichloroacetic acid (TCA). Protein (650 μg) was mixed with 5% TCA (final concentration) in 50 mM Na-phosphate buffer, pH 8, containing 300 mM NaCl, 5% glycerol and 0.3% LDAO. The mixture was briefly centrifuged and protein pellets were resuspended in 100 mM Na-phosphate buffer, pH 7, containing 1% sodium dodecyl sulfate (SDS). In some cases, sonication was needed to resuspend the pellet completely. Visible spectra of the resuspended pellet and supernatants were obtained to determine the amount of covalently bound flavin.

Fluorescence spectra of the purified proteins were obtained using a Fluoromax 2 (Instruments SA) fluorimeter exciting at 460 nm for the emission spectra and monitoring at 525 nm for the excitation spectra. The samples contained purified protein (NqrC, 60 μg/ml; H216L, 19 μg/ml; T225L, 19 μg/ml) in 100 mM Na-phosphate buffer, pH 7, with 1% SDS, 300 mM NaCl, and 5% glycerol.

2.5. Other methods

Protein concentration was determined using the BCA (bicichonic acid) protein assay kit from Pierce with bovine serum albumin as a standard.

The expression of the recombinant NqrC (wild-type and mutants) was checked by Western blotting using whole cells, membranes or purified protein. Anti-His (C-terminus) antibodies from Invitrogen were used for the immunoblot. SDS–PAGE was performed using 12.5% polyacrylamide precast gels (Protean II Ready gel) from Bio-Rad in Tris–HCl buffer. Gels were then stained using Gel-Code blue reagent and photographed under UV illumination using a Gel Doc 2000 gel documentation system from Bio-Rad. For Western blotting, proteins from an SDS gel were transferred to a polyvinylidenedifluoride membrane using a methanol–glycine transfer buffer. The alkaline phosphatase conjugate system was used to visualize the Western blots according to the instructions from the manufacturer (Bio-Rad).

3. Results

3.1. Expression of NqrC

The *nqrC* gene from *V. cholerae* was cloned into the pBAD expression vector. The pBAD system contains an arabinose-inducible promoter (*ara*BAD), and the vector used inserts a 6×-His tag at the C-terminus of the expressed protein. This *nqrC*-pBAD construct was then introduced into *V. cholerae* by electroporation. Arabinose-dependent *nqrC* expression was demonstrated by Western blotting with an antibody against the 6×-His tag. Although arabinose was found to be necessary for expression, the same levels of expression were obtained over a wide range of arabinose concentrations (from 0.00002 to 0.2%). The expression of *nqrC* does not have a negative effect on cell growth. The recombinant NqrC has a higher molecular weight than its genomic counterpart (32 kDa instead of 27.6 kDa) because of additional amino acids from the pBAD fusion construct. Hence, the recombinant NqrC expressed from the plasmid could be distinguished from the genome-encoded subunit on the basis of molecular weight, reactivity towards anti-His antibodies and by N-terminal sequencing (see below). Recombinant NqrC was purified using a Ni-NTA column. SDS–PAGE of the purified protein revealed two main bands (Fig. 1A): a strong band around 32 kDa and a less intense band around 70 kDa, which appears to be a dimer of NqrC that persists in the presence of SDS. Both bands (32 kDa and 70 kDa) were fluorescent under UV illumination (Fig. 1B), indicating that the flavin, normally found in the native NqrC, is also present in the recombinant protein. The N-terminal sequencing of the two bands showed that both have the sequence **GSGSGDDDKLALT<u>MAS</u>** where MAS is the start of the cloned NqrC and the preceding 13 residues come from the pBAD vector. It appears that under the conditions used NqrC forms a dimer which is stable after boiling in the presence of SDS and dithiothreitol. A Western blot using anti-His antibodies confirmed the presence of the 6×-His tag on the monomeric NqrC (Fig. 1C). The apparent dimer reacted weakly against the anti-His antibodies.

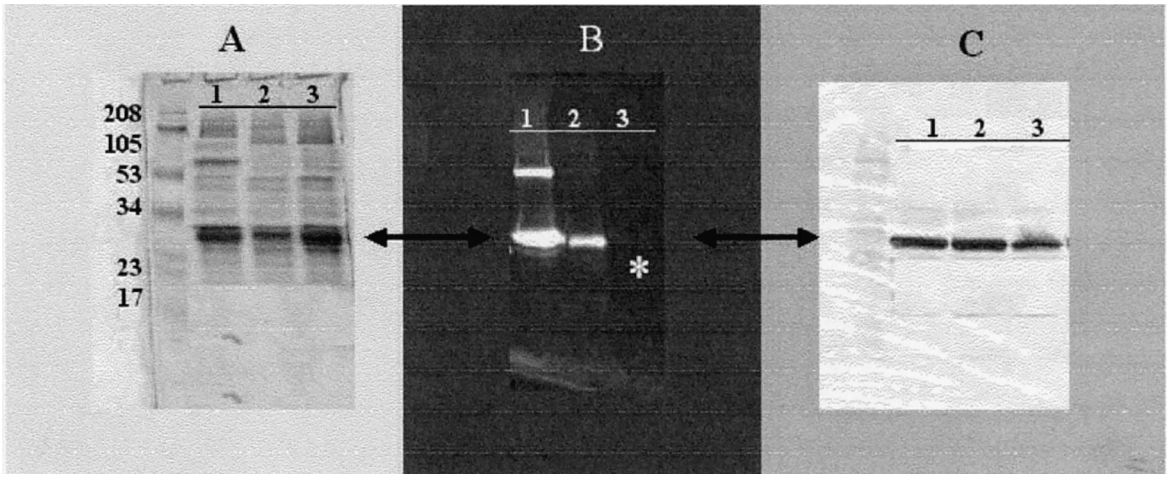

Fig. 1. SDS–PAGE of the purified recombinant NqrC (30 μg per lane): (1) wild-type, (2) H216L and (3) T225L proteins from *V. cholerae*. Three different gels (12% acrylamide, Tris–HCl system) are shown. A, Coomassie stain; B, UV illumination; and C, Western blot with anti-His tag antibodies.

3.2. Characterization of the purified protein

3.2.1. Visible spectroscopy. The oxidized-minus-reduced spectrum of the purified NqrC (Fig. 2) has a characteristic flavin absorbance at 453 nm. The peak is broad, with a shoulder near 470 nm and is similar to the spectra of other flavoproteins, e.g. trimethylamine dehydrogenase, which is covalently linked via a cysteine residue [17]. Other absorbance bands, apparently due to nickel contaminant from the column resin, were often observed, but these could be eliminated by dialyzing the enzyme against 10 mM EDTA.

The amount of flavin in the protein was calculated from the oxidized-minus-reduced spectrum using an extinction coefficient of 12 mM^{-1} cm^{-1} at 453 nm. The recombinant NqrC contains 0.8–1 mol of flavin per mol of protein and this ratio was unchanged upon precipitation of the protein with TCA.

The same expression vector was used to express *nqrC* in *E. coli*. Significant amounts of recombinant NqrC were produced, apparent by Western blot analysis and SDS–PAGE of whole cells. However, the band corresponding to NqrC was not fluorescent, indicating the absence of covalent flavin.

3.3. His-216 and Thr-225 mutations

The *nqrC*-pBAD system was used to study flavin attachment by site-directed mutagenesis. Mutants were constructed in which the two primary candidate residues, H216 and T225, were individually changed to leucine. Western blot analysis confirmed that both mutant proteins were expressed and incorporated in the cell membrane when arabinose was present in the growth medium. The H216L and T225L proteins were purified by the same protocol used for the wild-type NqrC. Fig. 1A shows a Coomassie-stained SDS gel of the purified proteins. A band around 32 kDa can be seen in the lane for each mutant. UV illumination of the gel (Fig. 1B) clearly shows that the H216L mutant protein is highly fluorescent, whereas the T225L mutant protein is not fluorescent. This is consistent with the identification of T225 as the flavin attachment site [14,15]. Note that, although the flavin is absent from the T225L mutant, the protein is expressed as efficiently as the wild-type NqrC. The attachment of the flavin is not necessary for the stability of the NqrC protein or for its localization in the membrane.

The purified H216L and T225L proteins were studied by UV and fluorescence spectroscopies. Fig. 2 shows the oxidized-minus-reduced visible spectra of the wild-type NqrC and the H216L mutant. The two spectra have the same line shape. Whereas the wild-type NqrC contains approximately 1 (0.8–1) mol of flavin per mol of protein, the H216L mutant contains 0.2–0.3 mol of flavin per mol of protein. This suggests that H216 may play a role in stabilizing the FMN bound to NqrC but is not essential for covalent attachment. The T225L mutant does not have any significant absorbance in the flavin region.

In order to confirm that the flavin in H216L is covalently attached, each purified protein was precipitated by TCA. Spectra of the dissolved pellets demonstrated the covalently bound flavin in both the wild-type and H216L NqrC proteins.

The flavin associated with the purified proteins was also studied by fluorescence spectroscopy after denaturation by SDS (Fig. 3). The relative fluorescence was normalized by

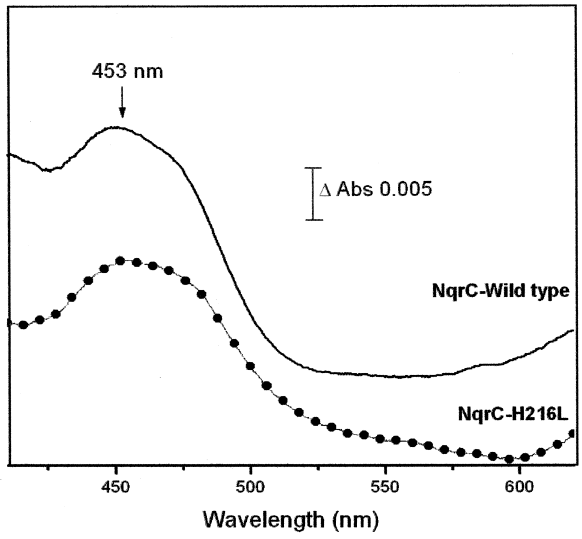

Fig. 2. Oxidized-minus-dithionite reduced difference absorption spectra of the purified wild-type NqrC (40 μg/ml) and the H216L mutant (190 μg/ml) (dotted line) from *V. cholerae*. The buffer used was 10 mM Na-phosphate pH 8, containing 300 mM NaCl, 0.3% LDAO, 5% glycerol, and 10 mM EDTA.

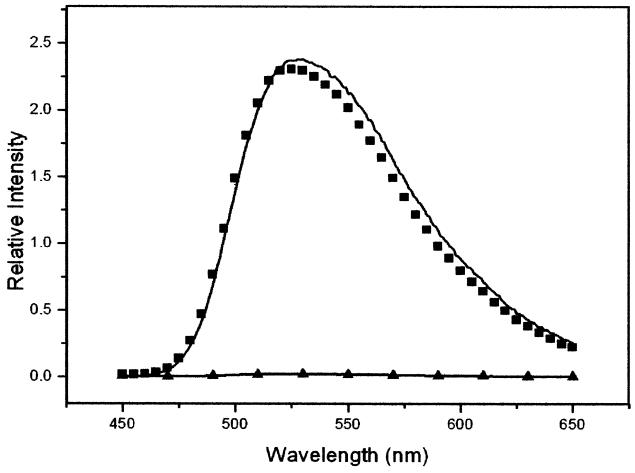

Fig. 3. Fluorescence emission (excitation at 460 nm) spectra of the wild-type NqrC (continuous line) and the H216L mutant (■) in 100 mM Na-phosphate, pH 7, with 1% SDS. The spectra were normalized to the amount of protein, and then the spectrum of H216L was multiplied by a factor of 3.3 to match the intensities. The fluorescence of the T225L mutant, normalized to the same amount of protein, is also shown (▲).

the protein concentration. The T225L mutant shows almost no fluorescence (< 1%), while the H216L mutant has about 30% of the fluorescence intensity compared to the wild-type NqrC. Fig. 3 also includes the spectrum of H216L mutant which has been multiplied by a factor of 3.3. The spectrum overlays on the emission spectrum of the wild-type. The data are consistent with the absorbance studies, indicating about 30% incorporation of the flavin in the H216L mutant. The T225L mutant exhibits a very small fluorescence, indicating that the protein incorporates a very small amount of flavin (< 1%). It is not known whether the trace amount of flavin in the T225L protein is covalently attached.

3.4. NqrB expression is toxic for V. cholerae

The NqrB subunit of the *V. alginolyticus* Na^+-NQR also contains a covalently bound flavin [14,15]. NqrB is a very hydrophobic protein and it was found that cell growth halted as soon as expression of cloned *nqrB* was induced by arabinose. Further studies using this approach were not undertaken.

4. Discussion

This is the first report of the genetic manipulation of a subunit of the respiratory sodium pump, Na^+-NQR, present in many pathogenic bacteria [4,5]. The *nqrC* gene from *V. cholerae* was cloned into a pBAD vector, expressed in *V. cholerae*, and the isolated recombinant protein was shown to contain covalently bound flavin. Flavin attachment does not depend upon incorporation of the NqrC subunit into the assembled enzyme. Although the *nqr* operon is present in the genome of the host strain of *V. cholerae*, the recombinant NqrC subunit is isolated as a single polypeptide.

The same *nqrC*-pBAD expression vector was also used in an *E. coli* host, but the resulting polypeptide did not contain covalent flavin. The reason for the lack of flavin binding to the recombinant NqrC expressed in *E. coli* is not clear. Possibly, the protein simply does not fold properly in *E. coli* under the conditions used, or perhaps the flavin attachment to threonine requires additional factors that are not present in *E. coli*. This will require further study. It is noted that all proteins previously characterized with covalently bound flavin, including several in *E. coli*, bind the cofactor via cysteine, histidine or tyrosine residues [18].

Two NqrC mutants, H216L and T225L, were also expressed in *V. cholerae*, purified and characterized. The results are consistent with T225 as the site of FMN attachment to the NqrC subunit of the *V. cholerae* sodium pump. The T225L mutation not only blocks covalent attachment of the flavin, but also prevents the incorporation of non-covalent FMN into the subunit. This is in contrast to the results obtained by mutagenesis of the covalent flavin attachment site in succinate dehydrogenase from *Saccharomyces cerevisiae*, for example, in which the flavin binds non-covalently to the protein despite the mutation [19]. The lack of FMN binding to the NqrC T225L mutant may be due to a direct interference at the flavin binding site but, alternatively, it could be an indirect effect due to a conformational change in the mutant protein.

The H216L mutation reduces the FMN content (30%) of the isolated NqrC, so it can be concluded that H216 is not essential for either flavin binding or covalent attachment. The H216L mutation, however, destabilizes the binding of FMN to the protein. The results show that the hydrophilic region including H216 and T225 is important for FMN binding the NqrC.

Future work will be directed at characterizing the Na^+-NQR complex from *V. cholerae* and exploring the role of the covalent flavin in the intraprotein electron transfer reactions and in the mechanism of sodium pumping.

Acknowledgements: We would like to thank Dr. Joel Morgan and Dr. Petra Hellwig for many discussions and suggestions. We also thank Dr. Theodore Hazlett at the University of Illinois Laboratory of Fluorescence Dynamics for his help with the fluorescence measurements. This work was supported by Grants from the Human Frontier Science Program and the National Institutes of Health (HL16101,RBG).

References

[1] Kogure, K. (1998) Curr. Opin. Biotechnol. 9, 278–282.
[2] Tokuda, H. and Unemoto, T. (1981) Biochem. Biophys. Res. Commun. 102, 265–271.
[3] Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014.
[4] Zhou, W., Bertsova, Y.V., Feng, B., Tsatsos, P., Verkhovskaya, M.L., Gennis, R.B., Bogachev, A.V. and Barquera, B. (1999) Biochemistry 38, 16246–16252.
[5] Häse, C.C. and Barquera, B. (2001) Biochim. Biophys. Acta, in press.
[6] Pfenninger-Li, X.D., Albracht, S.P.J., van Belzen, R. and Dimroth, P. (1996) Biochemistry 35, 6233–6242.
[7] Dibrov, P.A., Kostyrko, V.A., Lazarova, R.L., Skulachev, V.P. and Smirnova, I.A. (1986) Biochim. Biophys. Acta 850, 449–457.
[8] Bogachev, A.V., Murtazina, R.A. and Skulachev, V.P. (1997) FEBS Lett. 409, 475–477.
[9] Kojima, S., Yamamoto, K., Kawagishi, I. and Homma, M. (1999) J. Bacteriol. 181, 1927–1930.
[10] Rich, P.R., Meunier, B. and Ward, F.B. (1995) FEBS Lett. 375, 5–10.
[11] Nakayama, Y., Hayashi, M. and Unemoto, T. (1998) FEBS Lett. 422, 240–242.
[12] Pfenninger-Li, X.D. and Dimroth, P. (1995) FEBS Lett. 369, 173–176.
[13] Steuber, J., Krebs, W. and Dimroth, P. (1997) Eur. J. Biochem. 249, 770–776.

[14] Nakayama, Y., Yasui, M., Sugahara, K., Hayashi, M. and Unemoto, T. (2000) FEBS Lett. 23723, 1–4.
[15] Hayashi, M., Nakayama, Y., Yasui, M., Maeda, M., Furuishi, K. and Unemoto, T. (2000) FEBS Lett. 24493, 1–4.
[16] Häse, C.C. and Mekalanos, J.J. (1999) Proc. Natl. Acad. Sci. USA 96, 3183–3187.
[17] Mewies, M., Basran, J., Packman, L.C., Hille, R. and Scrutton, N.S. (1997) Biochemistry 36, 7162–7168.
[18] Mewies, M., McIntire, W.S. and Scrutton, N.S. (1998) Protein Sci. 7, 7–20.
[19] Robinson, K.M., Rothery, R.A., Wiener, J.H. and Lemire, B.D. (1994) Eur. J. Biochem. 222, 983–990.