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Gene fusions with β-lactamase show that subunit I of the cytochrome *bd* quinol oxidase from *E. coli* has nine transmembrane helices with the O_2 reactive site near the periplasmic surface

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Abstract The cytochrome *bd* quinol oxidase is a component of the respiratory chain of many prokaryotes. The enzyme contains two subunits, CydA and CydB, which were initially predicted based on the sequence of the *Escherichia coli* oxidase to have seven and eight transmembrane spans, respectively. More recently, the topological model of CydA was revised to predict nine transmembrane helices, based on additional sequence information from other organisms. In the current work, the topology of the *E. coli* oxidase was experimentally examined using β-lactamase gene fusions. The results confirm the revised topology, which places the oxygen reactive site near the periplasmic surface.

Key words: Oxidase; Cytochrome *bd*; Topology; Membrane protein; β-Lactamase; Gene fusion

1. Introduction

The cytochrome *bd* quinol oxidase is a component of the respiratory chains of many prokaryotes [1–3] and is often associated with growth under conditions of low aeration. Most prokaryotic quinol oxidases and cytochrome *c* oxidases belong to the heme-copper oxidase superfamily [4,5], but cytochrome *bd* is not part of this superfamily [1,3,6]. Mutations of cytochrome *bd* in both *Shigella flexneri* [7] and in *Brucella abortus* [8] result in decreased virulence, and the enzyme may play a role in protection against oxidative stress [9]. Since this oxidase is found only in bacteria, including a number of human pathogens, cytochrome *bd* may be of interest as a drug target.

The most extensively characterized cytochrome *bd* is the one isolated from *Escherichia coli* [6,10–13], where it is expressed maximally under microaerophilic growth conditions [14,15]. The enzyme consists of one copy each of two integral membrane subunits, CydA (subunit I) and CydB (subunit II), encoded by the *cyd*AB operon in *E. coli* [16,17]. The enzyme catalyzes the two-electron oxidation of ubiquinol-8 or menaquinol-8 in the bacterial membrane, and the four-electron reduction of dioxygen to water. The quinol oxidation results in the release of protons to the periplasm and the generation of a proton motive force [13,18–20]. The proton motive force results from the fact that the electrons and protons used in the chemistry of converting O_2 to water are delivered to the active site from opposite sides of the membrane.

The enzyme contains three heme prosthetic groups: heme b_{558}, heme b_{595} and heme *d*. Heme b_{558} has been implicated in the oxidation of ubiquinol, and the axial ligands have been identified as His186 [21] and Met393 [22] in subunit I. In both the initially proposed and revised topologies of subunit I, these residues are predicted to be near the periplasmic end of transmembrane helices [3,23]. The proposed locations of these residues are shown in the models depicted in Fig. 1A and B.

The active site where O_2 is reduced to water is a bimetallic center comprised of heme b_{595} and heme *d* [12,24,25]. O_2 binds to the ferrous iron of heme *d* [1,26,27], and the role of heme b_{595} [10] is not known. The axial ligand of heme b_{595} has been identified as His19 in subunit I [21,28]. According to the initially proposed topology of subunit I [23,29], this residue was predicted to be near the cytoplasmic side of the bacterial membrane (Fig. 1A), but the revised topology [3] places His19 near the periplasmic surface (Fig. 1B). According to the initially proposed topology (Fig. 1A), the major charge movement responsible for the generation of a transmembrane potential during turnover of cytochrome *bd* is electron transfer from heme b_{558} near the outer surface of the membrane to the heme b_{595}/heme *d* active site on the opposite side of the membrane (see Fig. 2B). In contrast, if His19 (i.e. heme b_{595} and heme *d*) is located near the periplasmic surface, then the membrane potential must result primarily from protons moving from the bacterial cytoplasm to the enzyme active site on the opposite side of the membrane (Fig. 2A). The current work confirms the revised topology and implies the existence of a proton-conducting channel required to convey protons from the bacterial cytoplasm to the enzyme active site.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli Top10 cells (Kan^R) (Invitrogen) were used in all the experiments. The plasmid pTK1 (Fig. 3), a derivative of pBR322, was the source of the DNA template for subcloning fragments of the *cyd*AB operon into the pBAD-TOPO vector (Invitrogen). This was used to amplify the DNA fragments, and then clone into the *Xba*I site of plasmid pWP101 (Kan^R) [30] to generate the gene fusion constructs (see Fig. 3).

2.2. Construction of gene fusions

Polymerase chain reaction (PCR) forward primers containing either the *Asc*I site (for cloning *cyd*A fragments) or the *Mlu*I site (for cloning

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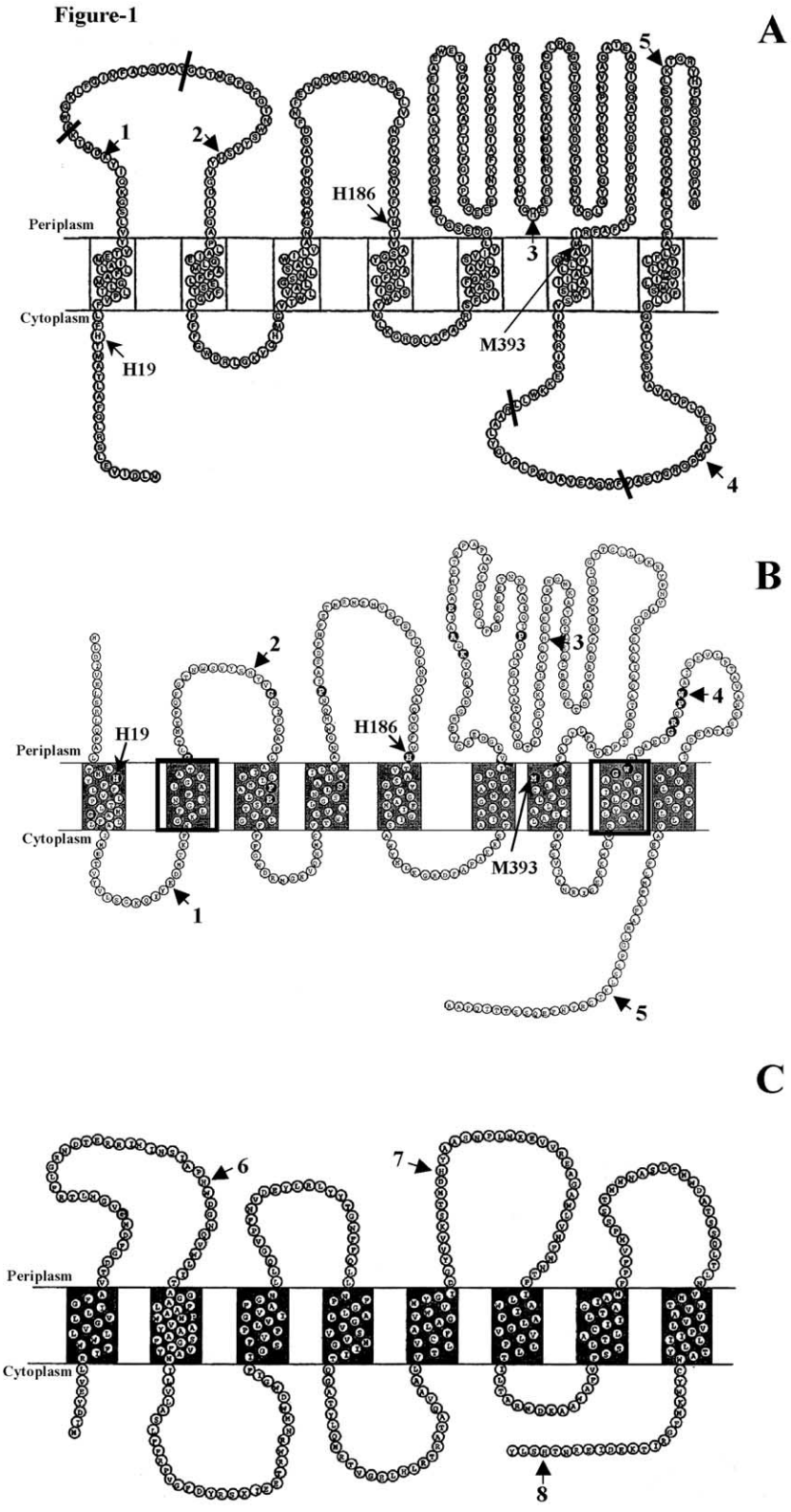

Fig. 1. Topological models of the subunits of the cytochrome *bd* quinol oxidase from *E. coli*. A: Previously proposed topology of CydA (subunit I) [23,29]. The three residues identified as heme axial ligands are indicated. The short dark lines indicate the boundaries of the transmembrane helices that are proposed by the revised topology, shown in B. B: The revised topology of CydA (subunit I) [3]. The black boxes indicate the two additional transmembrane helices. C: Topological model of CydB (subunit II) [3]. Arrows show the location of β-lactamase fusion junctions, numbered as referred to in Table 1.

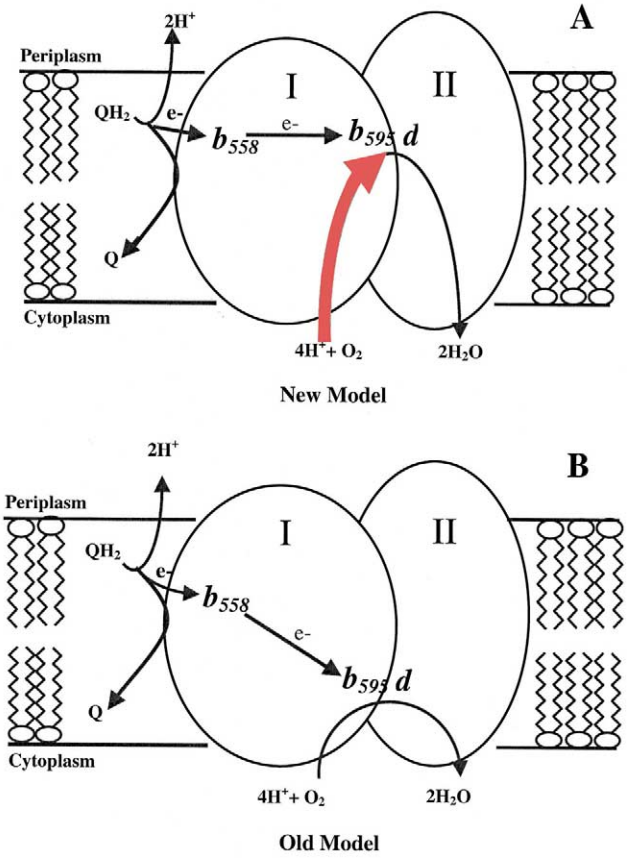

Fig. 2. Schematic models showing the two models in which the location of the heme b_{595}/heme *d* binuclear center is at the periplasmic (A) or cytoplasmic (B) side of the membrane. Electrons are presumed to be transferred from heme b_{558} to the heme b_{595}/heme *d* center, and protons used in the chemistry to generate H_2O from O_2 come from the cytoplasmic side of the membrane. The data in this paper favor the model shown in A, which requires a proton-conducting channel within the protein.

*cyd*B fragments) and reverse primers containing the *Xba*I site (Table 1) were used to amplify portions of the *cyd*A or *cyd*B starting from the N-termini using Taq polymerase (Invitrogen) (Fig. 3). The minicycler (MJ Research) program was set as follows: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, number of cycles: 30. PCR products were cleaned by agarose gel electrophoresis and cloned into the pBAD-TOPO vector; the constructs were then transformed into Top10 competent cells. Plasmid DNA from the pBAD-TOPO containing various portions of the *cyd* operon were digested using *Asc*I/*Xba*I restriction enzymes (for *cyd*A fusions) or *Mlu*I/*Xba*I (for *cyd*B fusions) (Fig. 3). The desired DNA fragments were isolated by agarose gel electrophoresis, extracted using GenElute columns (Sigma) by centrifuging at 13 000 rpm for 10 min, and then ligated to pWP101 vector, which had been digested with *Mlu*I/*Xba*I restriction enzymes (Fig. 3). The ligation reactions were used to transform Top10 competent cells and the colonies were selected on Luria–Bertani (LB) (kanamycin 50 μg/ml) plates. The fusion junctions of the resulting plasmid products were confirmed by DNA sequencing.

2.3. Plate tests of β-lactamase activity

A single colony of *E. coli* Top10 harboring the gene fusion plasmid (pWP101 derivative) was grown in LB-Kan (50 μg/ml) overnight, then diluted 10^6-fold. A 100 μl aliquot of each dilution was spread on LB plates containing 50 μg/ml kanamycin, and concentrations of ampicillin ranging from 5 to 1000 μg/ml. Colonies were counted after 24 h of growth at 37°C. Assays were repeated at least twice for each fusion with cultures starting from different individual colonies.

3. Results

In order to distinguish between the two models of CydA (Fig. 1A, B), five gene fusions were designed, in which selected N-terminal fragments from CydA was fused in-frame to β-lactamase. To confirm the methodology, three additional fusions were made to CydB, whose topology is not in question [23]. The locations of the fusion junctions are shown in Fig. 1, and the junction sequences are included in Table 1.

Fusions which place the β-lactamase on the periplasmic side of the membrane should be highly resistant to ampicillin. However, β-lactamase located in the bacterial cytoplasm will only have access to ampicillin, if the cells die and lyse. Living cells in the immediate vicinity of lysed cells will appear resistant to ampicillin, which will be manifested by patches of cells appearing when the plate is inoculated with a high cell density [31].

To verify the expression of functional β-lactamase for each fusion, a high-density inoculum ($\sim 10^6$ cells/inoculum) of *E. coli* culture was spread on LB plates containing a series of concentrations of ampicillin (5 to 1000 μg/ml). Cells expressing each of the gene fusions showed growth on plates containing at least 50 μg/ml of ampicillin. The maximum ampicillin concentration tolerated by each fusion construct is shown in Fig. 4. Those constructs that result in isolated patches of colonies [31] are presumed to be fusions where the β-lactamase is located on the cytoplasmic side of the membrane. The remaining fusions grew as lawns of cells on the ampicillin plates. All of the constructs exhibited resistance to ampicillin.

Table 1
Cyd/β-lactamase fusions

Fusion no.	Junction location	Junction sequence[a]	Predicted activity from old model[b]	Predicted activity from new model[b]	Maximum Amp conc. on which cells can grow (μg/ml)
CydA (subunit I)					
1	I-K49	CAG ATT TAT AAA TCT AGA CAC CCA	+	−	10
2	I-H86	TAC TAT TCC CAC TCT AGA CAC CCA	+	+	100
3	I-H314	ATG GTG CAG CAT TCT AGA CAC CCA	+	+	1000
4	I-W451	CGC CAA CCG TGG TCT AGA CAC CCA	−	+	1000
5	I-K505	AGC AGC CTG AAA TCT AGA CAC CCA	+	−	10
CydB (subunit II)					
6	II-H56	ATT GCA CCA CAC TCT AGA CAC CCA	+	+	500
7	II-H237	ACA ATG GAC CAT TCT AGA CAC CCA	+	+	500
8	II-H376	CGT AAC ACC CAC TCT AGA CAC CCA	−	−	10

[a]The sequence for β-lactamase is underlined.
[b]β-Lactamase activity, determined using growth on ampicillin plates.

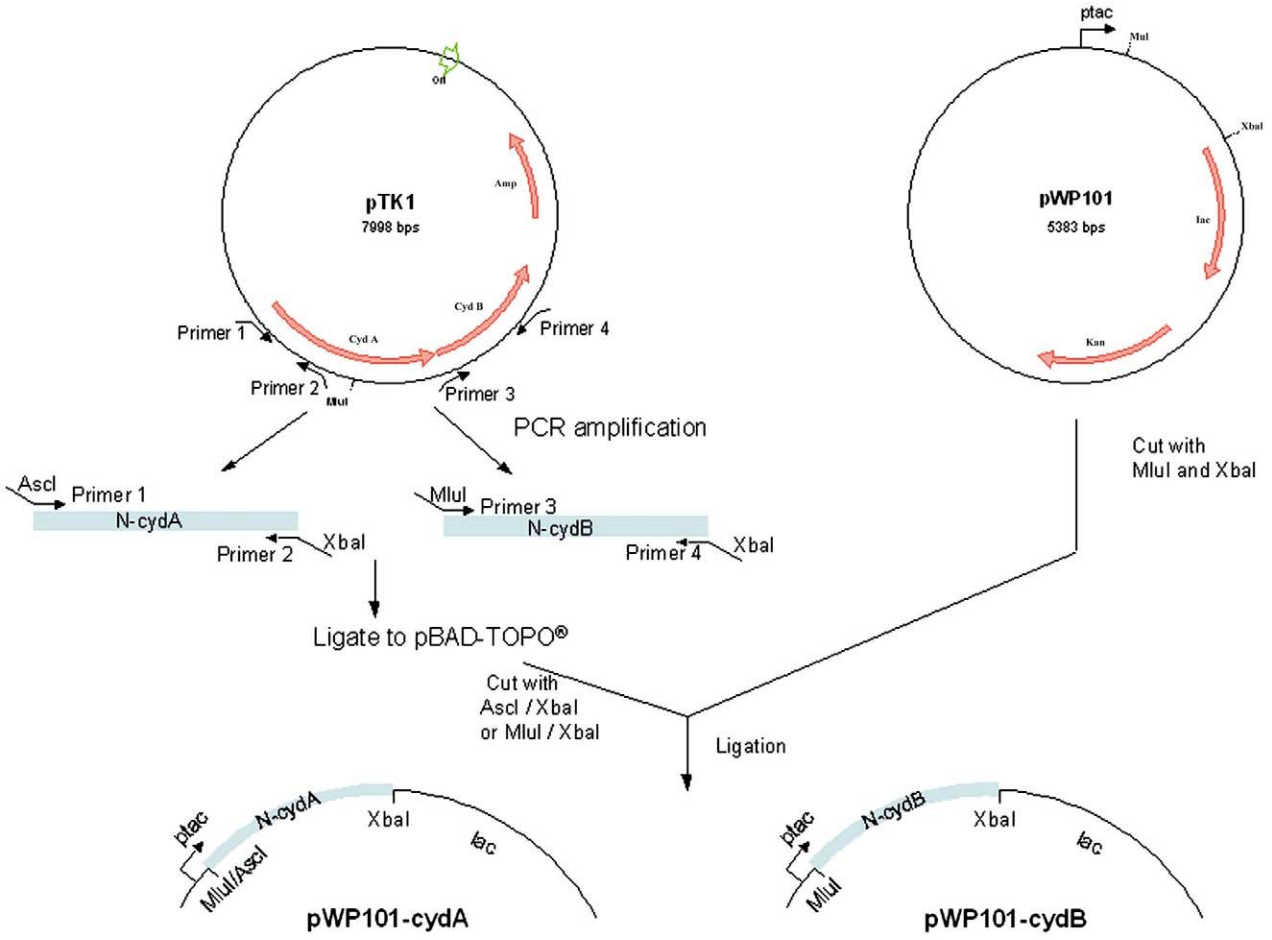

Fig. 3. Schematic showing the plasmids and constructs used to generate the gene fusions between either *cyd*A or *cyd*B and the gene encoding β-lactamase.

LB (Amp) plates were inoculated with a low density of cells (~200 cells/inoculum) to quantify the ampicillin sensitivity of each individual fusion construct. This also measures the accessibility of the β-lactamase moiety to extracellular ampicillin. By using a low-density inoculum, colonies are well separated on the plate, so there is no influence of lysed cells on the neighbors since the diffusion of ampicillin is limited. The strains expressing gene fusions with the β-lactamase moiety on the periplasmic surface are able to survive relatively high levels of ampicillin. The strains with β-lactamase retained in the cytoplasm are very sensitive to the drug, and 10 μg/ml of ampicillin is sufficient to kill all cells on the plate. Fig. 5 shows the maximum ampicillin concentrations allowing growth of each of the strains. Strains which are resistant to 100 μg/ml ampicillin or higher are interpreted as expressing fusions with the β-lactamase on the periplasmic side of the membrane.

4. Discussion

The results indicate that fusions 2, 3, 4, 6 and 7 are on the periplasmic side of the membrane and that fusions 1, 5 and 8 are on the cytoplasmic side. The three fusions within CydB (subunit II) are, as expected, consistent with the previously published data and with the hydrophobicity models [3,23]. The revised topology of CydA [3] includes two additional transmembrane spans that were not initially predicted [23]. These are boxed in Fig. 1B. The presence of these transmembrane spans [3] predicts that fusions 1 and 5 will be on the periplasmic side and that fusion 4 will be on the cytoplasmic side of the membrane, with the reverse true for the initially proposed topology [23]. As shown in Figs. 4 and 5 and summarized in Table 1, the experimental data are fully consistent with the new topology. Whereas the previously published gene fusion studies using alkaline phosphatase and β-galactosidase had gaps and ambiguities, the current data using β-lactamase fusions are clear. Note that both the old and the new topologies place the ligands to heme b_{558} (His186 and Met393) on the periplasmic side of the membrane (Fig. 1A, B). The 'Q

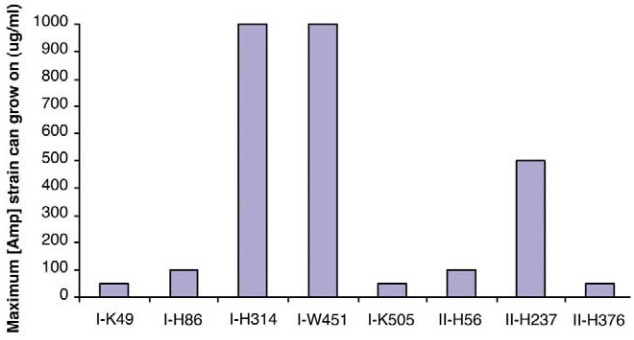

Fig. 4. Maximum ampicillin concentrations tolerated by cells expressing the cyd/β-lactamase gene fusions, using a high-density cell inoculum on LB/Amp plates.

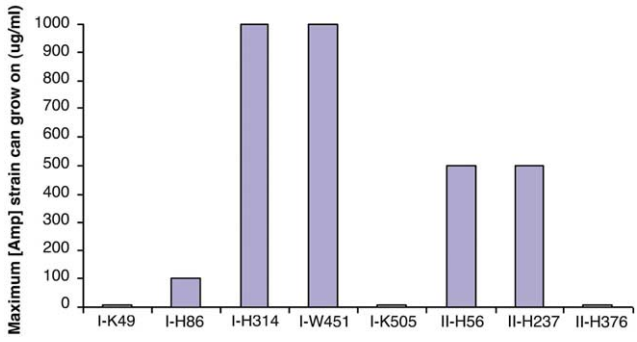

Fig. 5. Maximum ampicillin concentrations tolerated by the cyd/β-lactamase gene fusions, using a low-density cell inoculum on LB/Amp plates.

loop', implicated in quinol binding, has been previously identified as being on the periplasmic side of the membrane by proteolysis and antibody binding studies [32,33]. The data obtained with fusion 3 are consistent with this periplasmic location (Table 1).

The importance of the topological picture of CydA is that it predicts that His19 is located near the periplasmic surface of the protein. Since His19 has been identified as the ligand to the high spin heme b_{595} component of the oxidase [21,28], this places the heme b_{595}/heme d active site, where the O_2 chemistry is catalyzed, near the periplasmic surface of the membrane. Hence, all three hemes are predicted to be near the periplasmic surface, as depicted in the revised model in Fig. 2A. This suggests heme b_{558} and the heme b_{595}/heme d complex are likely to be close to each other. Furthermore, the electrogenic charge transfer across the membrane that is associated with turnover of the enzyme [13,18] must primarily be due to protons crossing the membrane from the cytoplasm to the active site on the periplasmic side of the membrane. If this is correct, there must be a proton-conducting channel to facilitate proton diffusion across the membrane (Fig. 2A). There are conserved acidic residues predicted to be within the membrane domain of CydA [3], which are logical candidates as residues that may contribute to such a channel. Future studies will explore this possibility.

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References

[1] Jünemann, S. (1997) Biochim. Biophys. Acta 1321, 107–127.
[2] Trumpower, B.L. and Gennis, R.B. (1994) Annu. Rev. Biochem. 63, 675–716.
[3] Osborne, J.P. and Gennis, R.B. (1999) Biochim. Biophys. Acta 1410, 32–50.
[4] Garcia-Horsman, J.A., Barquera, B., Rumbley, J., Ma, J. and Gennis, R.B. (1994) J. Bacteriol. 176, 5587–5600.
[5] Ferguson-Miller, S. and Babcock, G.T. (1996) Chem. Rev. 7, 2889–2907.
[6] Mogi, T., Tsubaki, M., Hori, H., Miyoshi, H., Nakamura, H. and Anraku, Y. (1998) J. Biochem. Mol. Biol. Biophys. 2, 79–110.
[7] Way, S.S., Sallustio, S., Magliozzo, R.S. and Goldberg, M.B. (1999) J. Bacteriol. 181, 1229–1237.
[8] Endley, S., McMurray, D. and Ficht, T.A. (2001) J. Bacteriol. 183, 2454–2462.
[9] Goldman, B.S., Gabbert, K.K. and Kranz, R.G. (1996) J. Bacteriol. 178, 6348–6351.
[10] Zhang, J., Hellwig, P., Osborne, J.P., Huang, H.-w., Moënne-Loccoz, P., Konstantinov, A.A. and Gennis, R.B. (2001) Bichemistry 40, 8548–8556.
[11] Yamazaki, Y., Kandori, H. and Mogi, T. (1999) J. Biochem. 125, 1131–1136.
[12] Borisov, V.B., Liebl, U., Rappaport, F., Martin, J.-L., Zhang, J., Gennis, R.B., Konstantinov, A.A. and Vos, M.H. (2002) Biochemistry 41, 1654–1662.
[13] Jasaitis, A., Borisov, V.B., Belevich, N.P., Morgan, J.E., Konstantinov, A.A. and Verkhovsky, M.I. (2000) Biochemistry 39, 13800–13809.
[14] Govantes, F., Albrecht, J.A. and Gunsalus, R.P. (2000) Mol. Microbiol. 37, 1456–1469.
[15] Govantes, F., Orjalo, A.V. and Gunsalus, R.P. (2000) Mol. Microbiol. 38, 1061–1073.
[16] Miller, M.J. and Gennis, R.B. (1983) J. Biol. Chem. 258, 9159–9165.
[17] Green, N.G., Fang, H., Lin, R.-J., Newton, G., Mather, M., Georgiou, C. and Gennis, R.B. (1988) J. Biol. Chem. 263, 13138–13143.
[18] Miller, M.J. and Gennis, R.B. (1985) J. Biol. Chem. 260, 14003–14008.
[19] Miller, M.J. and Gennis, R.B. (1986) Methods Enzymol. 126, 138–145.
[20] Gennis, R.B. (1987) FEMS Microbiol. Rev. 46, 387–399.
[21] Fang, H., Lin, R.-J. and Gennis, R.B. (1989) J. Biol. Chem. 264, 8026–8032.
[22] Kaysser, T.M., Ghaim, J.B., Georgiou, C. and Gennis, R.B. (1995) Biochemistry 34, 13491–13501.
[23] Newton, G., Yun, C.-H. and Gennis, R.B. (1991) Mol. Microbiol. 5, 2511–2518.
[24] Tsubaki, M., Hori, H., Mogi, T. and Anraku, Y. (1995) J. Biol. Chem. 270, 28565–28569.
[25] Hill, J.J., Alben, J.O. and Gennis, R.B. (1993) Proc. Natl. Acad. Sci. USA 90, 5863–5867.
[26] Kobayashi, K., Tagawa, S. and Mogi, T. (1999) Biochemistry 38, 5913–5917.
[27] Lorence, R.M., Koland, J.G. and Gennis, R.B. (1986) Biochemistry 25, 2314–2321.
[28] Sun, J. et al. (1996) Biochemistry 35, 2403–2412.
[29] Georgiou, C.D., Dueweke, T.J. and Gennis, R.B. (1988) J. Biol. Chem. 263, 13130–13137.
[30] Prinz, W.A. and Beckwith, J. (1994) J. Bacteriol. 176, 6410–6413.
[31] Wang, R.C., Seror, S.J., Blight, M., Pratt, J.M., Broome-Smith, J.K. and Holland, I.B. (1991) J. Mol. Biol. 217, 441–454.
[32] Dueweke, T.J. and Gennis, R.B. (1991) Biochemistry 30, 3401–3406.
[33] Dueweke, T.J. and Gennis, R.B. (1990) J. Biol. Chem. 265, 4273–4277.