The symbiotically essential cbb_3-type oxidase of *Bradyrhizobium japonicum* is a proton pump

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Abstract Purified cbb_3-type oxidase of *Bradyrhizobium japonicum* was reconstituted into phospholipid vesicles. Tight vesicles were obtained as shown by the disturbance of ΔpH with CCCP and the membrane potential with valinomycin, which led to a six-fold increase in cytochrome *c* oxidase activity. The vesicles were thus suitable for proton translocation experiments. In the presence of valinomycin, a pulse with reduced cytochrome *c* caused an acidification with a subsequent alkalinization, whereas the same pulse caused only an alkalinization in the presence of valinomycin plus CCCP. We conclude that the cbb_3-type oxidase of *B. japonicum* is a proton pump.

Key words: *Bradyrhizobium japonicum*; cbb_3-type oxidase; Cytochrome *c* oxidase; Nitrogen fixation; Proton pumping

1. Introduction

The cbb_3-type oxidase of the soybean symbiont *Bradyrhizobium japonicum* is essential for respiration when the bacterial cells live in endosymbiotic, nitrogen-fixing conditions within the root nodule [1,2,3]. It belongs to the superfamily of heme–copper oxidases [4] and is thought to transfer electrons from the cytochrome bc_1 complex to molecular oxygen. Such an electron transfer is usually coupled to the net translocation of protons from the cytoplasm to the periplasm. The resulting proton gradient is then used for the synthesis of ATP by ATP synthase [5,6].

Seven years ago, the cbb_3-type oxidase was discovered as a novel type of a cytochrome *c* oxidase as far as subunit composition and content of cofactors are concerned [1,7,8,9]. The membrane-integral FixN subunit (also called CcoN) resembles subunit I of classical heme-copper oxidases in having a low-spin heme and a binuclear center composed of a high-spin heme and Cu_B, where O_2 reduction to H_2O occurs. The heme and copper cofactors are liganded by six strictly conserved histidines [10]. The other redox-active, membrane-anchored subunits FixO (CcoO) and FixP (CcoP) are mono- and diheme *c*-type cytochromes, respectively. A fourth, hydrophobic subunit (FixQ, CcoQ) is firmly attached to the oxidase but has no obvious function [10]. All four proteins are encoded by the *fixNOQP* operon [1].

With a K_M of 7 nM, the cbb_3-type oxidase has an extremely high affinity for oxygen [2], allowing *B. japonicum* to respire in the micro-oxic environment within the root nodule. Although this species possesses a branched respiratory chain with at least four terminal oxidases [2,11,12,13], the cbb_3-type oxidase is the only one that supports symbiotic nitrogen fixation. This oxidase's function is either to scavenge oxygen in order to protect nitrogenase from irreversible oxidative damage, or to act as a proton pump which, coupled with oxidative phosphorylation, would help meet the high ATP demands of the nitrogenase reaction [14]. Here, we report the in vitro reconstitution of purified cbb_3-type oxidase in the form of proteoliposomes and demonstrate proton pumping activity. Hence, this oxidase plays a pivotal role in energy conservation for nitrogen fixation in symbiosis.

2. Materials and methods

2.1. Overexpression of the B. japonicum cbb_3 oxidase

To enhance expression of the cbb_3-type oxidase, the $fixN^{His}OQP$ genes were cloned into the broad host-range plasmid pPP375Ω-1 [15,16] derived from pRK290. This plasmid was linearized with *Bam*HI and the 5′ overhangs were filled with T4 DNA polymerase. A *Kpn*I linker was ligated to this blunt-end site (pRJ4662). The $fixN^{His}OQP$ operon was excized with *Kpn*I from pRJ4620 [10,16] and ligated into *Kpn*I-digested pRJ4662, resulting in pRJ4639. This plasmid was conjugated into *B. japonicum* strain Bj4621 [10,16], yielding strain Bj4639.

2.2. Enzyme preparation and activity measurement

The *Paracoccus denitrificans* aa_3-type oxidase was purified as described [17]. The cbb_3-type oxidase was isolated from membranes of *B. japonicum* cells grown anaerobically with nitrate as described previously [3] except for the following modifications: the membranes were isolated from cells disrupted in the presence of 0.15 mg lysozyme per ml and washed in 20 mM Tris–HCl, pH 8, 1 mM PMSF at 4°C. Chromatographic columns were equilibrated with the same buffer as described previously [10], but with 10% (v/v) glycerol added. The entire purification procedure was performed at 4°C. Buffer exchange against the reconstitution buffer (100 mM HEPES–KOH, 10 mM KCl, pH 7.3) was performed on a gel filtration column. The enzyme preparation was concentrated by ultrafiltration with 100-kDa cut-off Amicon centricons up to a concentration of 190 μM (24 mg/ml). Oxidase concentration was measured spectrophotometrically by recording the absorption difference between Na-dithionite-reduced and air-oxidized samples at 551 nm [10], using an extinction coefficient of 19 cm^{-1} mM^{-1} for one heme C. Enzyme activity was calculated by measuring the change in absorbance at 550 nm of reduced horse heart cytochrome *c* during its oxidation by the cbb_3-oxidase at 25°C in 10 mM HEPES–KOH, 40 mM KCl, 40 mM sucrose, 0.01% (w/v) dodecylmaltoside. The preparation was stored at 4°C.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; Covs, cytochrome *c* oxidase vesicles; PMSF, phenylmethylsulfonyl fluoride; RCR, respiratory control ratio

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2.3. Preparation of proteoliposomes

L-α-Phosphatidylcholine (type IVS) from soybean was purified and stored as a stock-solution (140 mg/ml) in chloroform at −20°C [18]. Before preparation of the vesicles, L-α-phosphatidylcholine was dried at 4°C for 1 h under a stream of argon [19]. The dried fraction was then solubilized in reconstitution buffer containing 1.5% (w/v) cholate, to give a final concentration of 62.5 mg/ml, by gentle stirring at 4°C for 2 h under argon. The suspension was sonicated with 30-s pulses (maximum 40 W), using a Branson tip-sonifier B12, followed by 30-s incubations at 4°C until clarity was reached. The sonicated liposomes were centrifuged at 20 000×*g* and 4°C for 20 min. The *cbb*$_3$-type oxidase was pre-incubated in 1.5% (w/v) cholate for 15 min at 4°C before it was added to the liposomes to give a final concentration of 4 μM, which corresponds to 1:125 protein:lipid ratio [20]. The mixture was then dialyzed for 5 h against 500 volumes of the reconstitution buffer without cholate, twice (12 h each) against 500 volumes of 10 mM HEPES–KOH, 50 mM KCl, 50 mM sucrose, pH 7.3 and, finally, twice (12 h each) against 500 volumes of 10 μM HEPES–KOH, 55 mM KCl, 55 mM sucrose, pH 7.3. Dialysis was performed in a Pierce Slide-A-Lyzer^R cassette, with a 10-kDa cut-off at 4°C. The proteoliposomes were centrifuged at 15 000×*g* at 4°C. The supernatant was used for RCR (respiratory control ratio) estimations, right-side-out determinations and for proton pump experiments.

2.4. RCR estimations and right-side-out determination

The RCR estimation [21,22] was performed in 1 ml of the second dialysis buffer (see above) at 25°C. The change of absorbance of reduced horse heart cytochrome *c* was recorded at 550 nm in a Hitachi U3000 double-beam UV–VIS scanning spectrophotometer. In the presence of 50 μM reduced horse heart cytochrome *c*, a baseline for auto-oxidation was recorded. After 10 s, 20 μl of a 1:50 diluted proteoliposome solution was added. The activities in the coupled and uncoupled forms, i.e. without and with valinomycin/nigericin and CCCP, respectively, were expressed as a quotient to give the RCR values [21,22]. The final concentrations in the assay were 10 μM of CCCP and 5 μM of either valinomycin or nigericin, each added from ethanol stock solutions [23]. Addition of identical amounts of ethanol did not alter the activity. The fraction of right-side-out vesicles was determined by the detergent method [24] with 0.1% (w/v) dodecylmaltoside.

2.5. Proton pump experiment

The proton pump experiment was performed in a thermostated 2-ml glass vessel at 25°C. The pH change was measured with an Inlab^{R}410 pH-electrode from Mettler-Toledo and a Knick 766 Calimatic^R pH-meter. The pH was recorded with a Philips PM8261 Xt-recorder. The concentration of proteoliposomes was 2 μM in the experimental setup. After incubation with 5 μM valinomycin [25] for 3 min, the pH was adjusted to 7.3 with traces of 10 mM KOH or 10 mM HCl. The reduced horse heart cytochrome *c* solution was also adjusted to the same pH. Calibration was done by addition of known amounts of HCl. After recording of a base line, proteoliposomes were pulsed with reduced horse heart cytochrome *c* (16 nmol). Upon complete uncoupling of the proteoliposomes with 10 μM CCCP [25], a new calibration with KOH was performed and the pH was again adjusted with traces of KOH and HCl. The proteoliposomes were then pulsed again with the same amount of reduced horse heart cytochrome *c*. To determine the proton pump and water formation activities the signals were compared with the calibration bars [20].

Table 1
Characteristics of reconstituted cytochrome *c* oxidases

	B. japonicum cbb$_3$	*P. denitrificans aa*$_3$
Turnover[a]	90 – 100 e^-/s	320 e^-/s [17]
RCR	5.5 – 6	4.3 – 4.5
H^+ pumping	0.2 – 0.4 H^+/e^-	0.5 – 0.6 H^+/e^-
H^+ consumption[b]	0.9 – 1 H^+/e^-	0.9 – 1 H^+/e^-

[a]Oxidase solubilized in non-ionic detergent.
[b]Water formation.

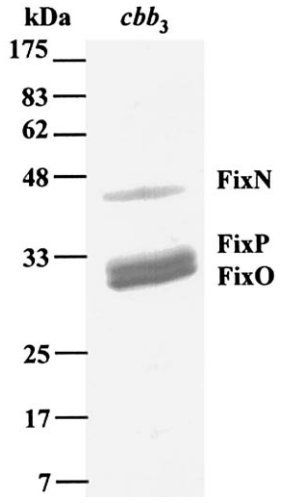

Fig. 1. SDS–PAGE of the purified *cbb*$_3$-type oxidase (10 μg). Protein subunits were visualized by silver stain. The apparent molecular masses of marker proteins are indicated on the left.

3. Results and discussion

In order to perform proton pumping experiments with proteoliposomes is was necessary to prepare *cbb*$_3$-type oxidase from *B. japonicum* in mg quantity. This was achieved by expression of a modified *fixNOQP* plasmid that led to synthesis of an oxidase carrying the histidine-tagged FixN. Subsequent purification included Ni-NTA affinity chromatography which proved to be effective for increasing the amount of oxidase that was solubilized from membranes. Fig. 1 shows a silver-stained SDS–polyacrylamide gel [26] of the purified oxidase. FixN, the largest subunit migrates at ∼45 kDa [10]. The diheme *c*-type cytochrome FixP and the monoheme *c*-type cytochrome FixO are detected at the 32- and 28-kDa positions, respectively. FixO and FixP were also detected by heme staining (not shown). With the improved oxidase expression and purification system the yield of oxidase was doubled (2.7 mg oxidase from 6 g wet cells) and oxidase activity with reduced horse heart cytochrome *c* as substrate (90 to 100 e^-/s; Table 1) was four times higher as compared with previously obtained values [10]. The reduced *cbb*$_3$-type oxidase (Fig. 2) showed absorption maxima in the α region characteristic for *c*- and *b*- type cytochromes [10].

Based on established procedures worked out with the *aa*$_3$-type cytochrome *c* oxidase of *P. denitrificans* [20], we developed a reconstitution procedure for the *B. japonicum cbb*$_3$-type oxidase. Estimates of the proportion of right-side-out vesicles revealed that 70–80% of the vesicles contained the embedded oxidase with the cytochrome *c* binding site facing outwards. As illustrated in Fig. 3a, the oxidase activities of coupled versus uncoupled proteoliposomes differed by a factor of 5.5 to 6 (RCR value). For comparison, and to control our experimental system, the RCR value of proteoliposomes containing *P. denitrificans aa*$_3$-type oxidase was also determined and shown to be 4.3 to 4.5 (Table 1). Next, we investigated the contribution of the ΔpH and the membrane potential to the coupled activity. Fig. 3b shows that abolishing the ΔpH with a protonophor (CCCP) after 30 s of oxidation led to a two-fold increased activity. After disruption of the membrane potential with valinomycin, a further 2.5- to 3-fold increase in activity was observed. From these experiments it is

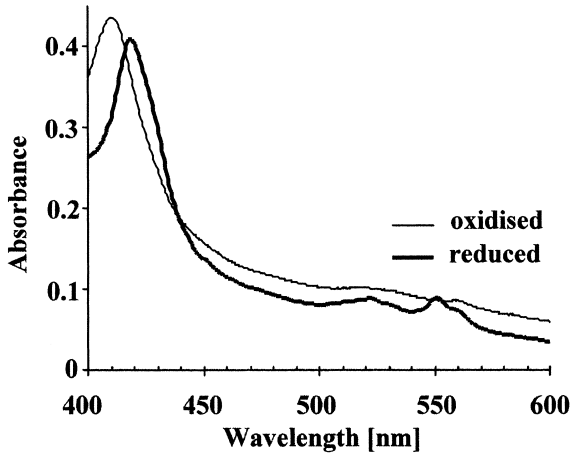

Fig. 2. Visible absorption spectra of 10 μg air-oxidized (thin line) and dithionite-reduced (thick line) cbb_3-type oxidase.

evident that both a protonophor and an ionophor are responsible for the enhanced activity. We conclude that the proteoliposomes were relatively tight in terms of their permeability for protons and potassium ions. Horse heart cytochrome *c* was neither oxidized by blank liposomes nor by proteoliposomes pre-incubated with KCN (not shown). Thus, we conclude that the cbb_3-type oxidase embedded into liposomes can couple electron flow to the generation of an electrochemical proton gradient.

To show whether protons are translocated across the vesicular membranes, we performed a proton pump experiment with a pH electrode. The observations are documented in Fig. 4. The experiment was performed in the presence of valinomycin to avoid a membrane potential that counteracts the proton extrusion. Upon a pulse of reduced horse heart cytochrome *c* to the proteoliposomes, a relatively rapid and pronounced acidification was observed. Following the immediate decrease of pH in the surrounding medium, an alkalinization back to the original pH level occurred. This alkalinization progressed further because of proton consumption inside the vesicles for the formation of H_2O. Addition of CCCP disrupted ΔpH and under this condition (with the same pulse of horse heart cytochrome *c*) only water formation was observed (Fig. 4). As a control, the same experiment was performed with the aa_3-type oxidase of *P. denitrificans*, which is a well-known proton pump [20]. The stoichiometries of proton pumping and proton consumption for water formation were calculated from these experiments and are listed in Table 1. With similar RCR values and ~ 1 H^+/e^- for water formation, the reconstituted cbb_3-type oxidase had a substantially lower proton pumping activity (0.2 H^+/e^- to 0.4 H^+/e^-) than the reconstituted aa_3-type oxidase. Moreover, water formation in the case of the cbb_3-type oxidase was always slower (Fig. 4) than that observed with the aa_3-type oxidase (not shown). We do not have a reasonable explanation for this behavior.

The lower level of proton translocation observed with the cbb_3 oxidase as compared with cytochrome aa_3 may be due to the slow turnover of the enzyme (Table 1). Perhaps, horse heart cytochrome *c* is not an ideal substrate for this oxidase which, in vivo, may obtain electrons from cytochrome c_1 as the natural substrate. A low proton pumping efficiency was also shown recently for the cbb_3-type oxidase of *Helicobacter pylori* [27], although precise values were not reported. Likewise, the in vivo proton pumping of the cbb_3-type oxidase of *P. denitrificans* was two times lower than that of the aa_3-type of the same organism [28]. In *Rhodobacter sphaeroides*, an oxygen pulse experiment with a strain suffering multiple deletions of genes encoding alternative terminal oxidases, leaving the cbb_3-type oxidase as the solely remaining functional oxidase, a proton pumping activity with a value of 1 H^+/e^- has been reported [29].

As mentioned, the cbb_3-type oxidase of *B. japonicum* has a very high affinity for oxygen. In symbiotic conditions, the proton pump must support energy conservation under extremely low O_2 tensions and thus might be slow. When compared with the classical proton-pumping heme–copper oxidase such as cytochrome aa_3, it is obvious that several of the conserved amino acid side chains of subunit I believed to form the proton translocating channels [30] are missing in FixN [10,28]. In the *P. denitrificans* aa_3-type oxidase the so-called K-channel consists of Ser^{291}, Lys^{354}, Thr^{351} and Tyr^{280} and directs protons from the cytoplasm to the binuclear center. The second, so-called D-channel consists of Asp^{124}, Asn^{113},

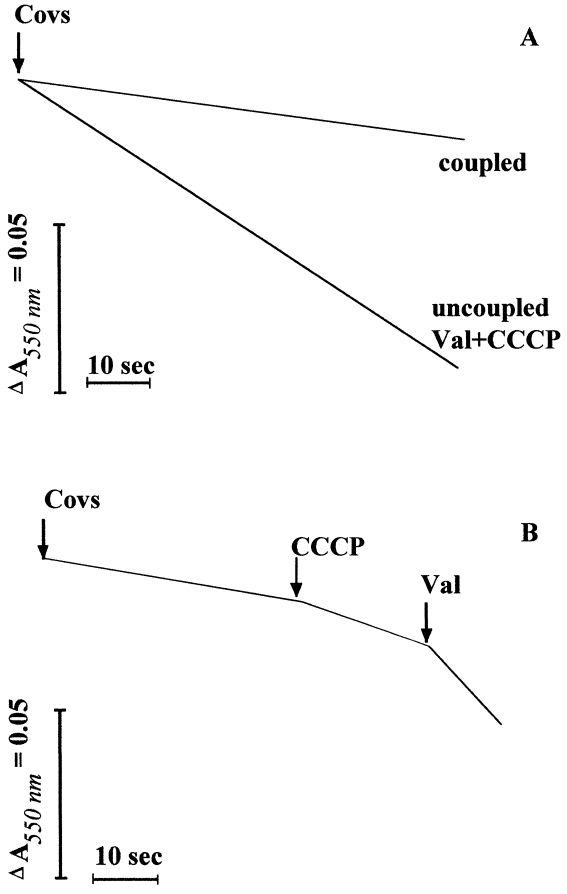

Fig. 3. Oxidation of horse heart cytochrome *c* by the cbb_3-type oxidase reconstituted in proteoliposomes. The reaction was started by addition of Covs containing 1.6 μmol oxidase. (A) The coupled state was monitored in the presence of 0.2% (v/v) ethanol. The uncoupled state was monitored after addition of 5 μM valinomycin (Val) and 10 μM CCCP, each dissolved in ethanol. (B) The same experimental setup as in panel A was used (without 0.2% (v/v) ethanol). After 30 s of oxidation the established ΔpH across the membrane was abolished by addition of 10 μM CCCP. After another 20 s, the membrane potential was disrupted completely with 5 μM valinomycin.

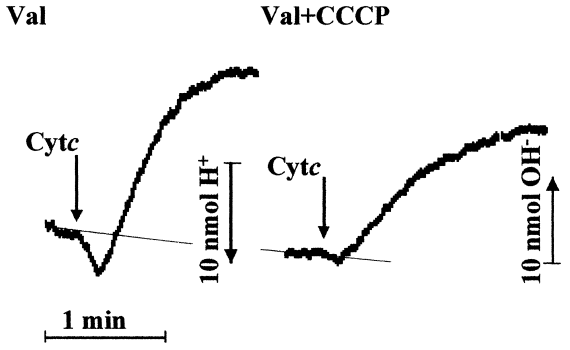

Fig. 4. Proton pump experiment with 2 nmol reconstituted *B. japonicum* cbb_3-type oxidase. In the presence of 5 μM valinomycin (Val), immediately upon a pulse of 16 nmol of horse heart cytochrome *c* (arrow), an acidification and subsequent alkalinization of the surrounding buffer occurred. Addition of 10 μM CCCP to the probe and again pulsing with the same amount of horse heart cytochrome *c* caused only an alkalinization.

Asn^{131}, Asn^{199}, Tyr^{35}, Ser^{134}, ser^{193} and Glu^{278} is believed to be for proton extrusion [30,31]. The residues corresponding to Asp^{124} and Glu^{278} are crucial for the proton pump activity of the bo_3 oxidase of *Escherichia coli* [32,33]. Therefore, despite the similar set-up of cofactors for redox chemistry, the cbb_3-type oxidase possible uses a different route for proton translocation than the classical heme-copper oxidases. A similar suggestion has been made for some archaeal terminal oxidases [31,34]

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References

[1] Preisig, O., Anthamatten, D. and Hennecke, H. (1993) Proc. Natl. Acad. Sci. USA 90, 3309–3313.
[2] Preisig, O., Zufferey, R., Thöny-Meyer, L., Appleby, C.A. and Hennecke, H. (1996) J. Bacteriol. 178, 1532–1538.
[3] Zufferey, R., Preisig, O., Hennecke, H. and Thöny-Meyer, L. (1996) J. Biol. Chem. 271, 9114–9119.
[4] Saraste, M. (1990) Q. Rev. Biophys. 23, 331–366.
[5] Nicholls, D.G. and Ferguson, S.J. (1992) Bioenergetics 2, Academic Press, London.
[6] Saraste, M. (1999) Science 283, 1488–1493.
[7] Garcia-Horsman, J.A., Berry, E., Shapleigh, J.P., Alben, J.O. and Gennis, R.B. (1994) Biochemistry 33, 3113–3119.
[8] Gray, K.A., Grooms, M., Myllykallio, H., Moomaw, C., Slaughter, C. and Daldal, F. (1994) Biochemistry 33, 3120–3127.
[9] Garcia-Horsman, J.A., Barquera, B., Rumbley, J., Ma, J. and Gennis, R.B. (1994) J. Bacteriol. 176, 5587–5600.
[10] Zufferey, R., Arslan, E., Thöny-Meyer, L. and Hennecke, H. (1998) J. Biol. Chem. 273, 6452–6459.
[11] Bott, M., Bolliger, M. and Hennecke, H. (1990) Mol. Microbiol. 4, 2147–2157.
[12] Bott, M., Preisig, O. and Hennecke, H. (1992) Arch. Microbiol. 158, 335–343.
[13] Surpin, M.A., Lübben, M. and Maier, R.J. (1996) Gene 183, 201–206.
[14] Hill, S. (1992) Physiology of nitrogen fixation in free-living heterotrophs, in: Biological Nitrogen Fixation (Stacey, G. and Burris, R.H., Eds.), pp. 87–134. Chapman and Hall, New York.
[15] Ramseier, T.M., Winteler, H.V. and Hennecke, H. (1991) J. Biol. Chem. 266, 7793–7803.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[17] Kleymann, G., Ostermeier, C., Ludwig, B., Skerra, A. and Michel, H. (1995) Bio/Technology 13, 155–160.
[18] Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5480.
[19] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[20] Solioz, M., Carafoli, E. and Ludwig, B. (1981) J. Biol. Chem. 257, 1579–1582.
[21] Casey, R.P. (1986) Methods Enzymol. 126, 13–21.
[22] Müller, M., Thelen, M., O'Shea, P. and Azzi, A. (1986) Methods Enzymol. 126, 78–87.
[23] Brown, G.C. and Cooper, C.E. (1995) Bioenergetics. A Practical Approach, IRL Press, Oxford.
[24] Carroll, R.C. and Racker, E. (1977) J. Biol. Chem. 252, 6981–6982.
[25] Sarti, P., Jones, M.G., Antonini, G., Malatesta, F., Colosimo, A., Wilson, M.T. and Brunori, M. (1985) Proc. Natl. Acad. Sci. USA 82, 4876–4880.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Tsukita, S., Koyanagi, S., Nagata, K., Koizuka, H., Akashi, H., Shimoyama, T., Tamura, T. and Sone, N. (1999) J. Biochem. 125, 194–201.
[28] De Gier, J.W.L., Schepper, M., Reijnders, W.N.M., van Dyck, S.J., Slotboom, D.J., Warne, A., Saraste, M., Krab, K., Finel, M., Stouthammer, A.H., van Spanning, R.J.M. and van der Oost, J. (1996) Mol. Microbiol. 20, 1247–1260.
[29] Toledo-Cuevas, M., Barquera, B., Gennis, R.B., Wikström, M. and Garcia-Horsman, J.A. (1998) Biochim. Biophys. Acta 1365, 421–434.
[30] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660–669.
[31] Schäfer, G., Engelhard, M. and Müller, V. (1999) Microbiol. Mol. Biol. Rev. 63, 570–620.
[32] Verkhovskaya, M.L., Garcia-Horsman, A.J., Puustinen, A., Rigaud, J.L., Morgan, J.E., Verkhovsky, M.I. and Wikström, M. (1997) Proc. Natl. Acad. Sci. USA 94, 10128–10131.
[33] Thomas, J.W., Puustinen, A., Alben, J.O., Gennis, R.B. and Wikström, M. (1993) Biochemistry 32, 10923–10928.
[34] Gleissner, M., Elferink, M.G.L., Driessen, A.J.M., Konings, W.N., Anemüller, S. and Schäfer, G. (1994) Eur. J. Biochem. 224, 983–990.