Cytochrome *c* Oxidase of the Cyanobacterium *Phormidium foveolarum*

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Z. Naturforsch. **44c,** 378–383 (1989); received January 16, 1989

Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Cytochrome aa_3, Cytochrome *c*-550, Cyanobacteria, *Phormidium foveolarum*

Phormidium foveolarum was grown with nitrate or ammonia as nitrogen source. With ammonia present respiration increased by a factor of 4 due to increase of cytochrome *c* oxidase. The inhibition of respiration by cyanide was measured during the growth period, indicating that only *one* terminal oxidase is present in *Phormidium foveolarum*. Cytochrome *c* oxidase from ammonia-grown filaments was solubilized and purified on phenylsuperose and by anion exchange 100-fold compared to the crude cell extract. The difference spectrum (oxidized minus reduced) shows absorption peaks at 417 nm, 514–520 nm, 550 nm and 605 nm. These maxima, together with a low inhibition constant for cyanide ($K_i = 0.4$ μM) and a higher one for azide ($K_i = 1.75$ mM) are interpreted in terms of an aa_3-type cytochrome *c* oxidase present containing noncovalently-bound cytochrome *c*.

Introduction

Prokaryotic cytochrome *c* oxidase may be used as a model since it is less complex than the mammalian enzyme [1]. Its simpler structural feature in prokaryotes is offset by metabolic complications. Most prokaryotes contain a branched electron transport system with more than one terminal oxidase [2] which may vary as a function of growth phase [3] or under different physiological conditions [4].

In cyanobacteria little is known on physiological influences and on molecular properties of cytochrome *c* oxidase. Due to the high content of photosynthetic pigments (chlorophylls and carotenoids) a spectral characterization of cytochrome *c* oxidase(s) is impossible without purifying the enzyme. The purification is difficult because of the instability of the enzyme, low cellular respiration rates and high pigment levels of the filaments. Furthermore, the cytochrome-oxidase content could not be stimulated significantly by exogenous substrates [5] but by elevating the Na^+-concentration in the growth medium [6]. Hitherto, only for one cyanobacterial species *(Anabaena variabilis)* purification of cytochrome *c* oxidase has been proven being of the aa_3-type [7]. Peschek and coworkers reported evidence for an aa_3-type oxidase present in *Anacystis nidulans* based on difference absorbance of chlorophyll-depleted membranes [8] and partially purified extracts [9]; the occurrence of other oxidases different from the aa_3-type was not excluded.

Respiration and cytochrome-oxidase content of *P. foveolarum* are stimulated by ammonia, and these filaments were used in this study to partially purify cytochrome *c* oxidase and to characterize it by difference spectra and inhibitor studies.

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0341–0382/89/0500–0378 $ 01.30/0

Materials and Methods

Preparation of cytochrome c oxidase

Axenic *Phormidium foveolarum* (No. B 1462-1, Algae Collection of Göttingen University) was autotrophically grown in batch cultures in nitrate-containing medium according to [7]. In ammonia-grown cultures nitrate was replaced by NH_4Cl ($0.107\ g \times l^{-1}$), and the phosphate concentration increased five-fold. Filaments from ammonia-grown cultures were used for enzyme preparation. After washing with buffer (A) containing 50 mM Tris-HCl, 2 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF), pH 7.5, filaments (ca. 20 ml, 1.5 mg chlorophyll (Chl) $\times$ ml^{-1}) were resuspended in buffer (A) and passed through a French-pressure cell (Aminco, Mod. J4-3339) at 110 MPa. Washed membranes (obtained by centrifugation for 45 min at $180{,}000 \times g$) were resuspended in buffer (B) (Tris-HCl 40 mM, EDTA 1 mM, pH 7.3). To solubilize cytochrome *c* oxidase 10 ml of the thylakoid suspension (equivalent to 1 mg Chl $\times$ ml^{-1}) were mixed with

detergent stock solutions (10% detergent, w/v) to achieve a final concentration of 1% octyl-β-D-thioglucoside (Calbiochem, Frankfurt, F.R.G.) and 0.2% deoxycholate (Sigma, Deisenhofen, F.R.G.), respectively. The suspension was incubated at 4 °C for 2 h without stirring and centrifuged at 450,000 × *g* for 90 min.

0.35 ml of crude solubilized enzyme was used for gel filtration on a FPLC Superose 6 column (Pharmacia, Uppsala, Sweden), equilibrated with buffer (B) containing 0.2% (w/v) deoxycholate. Fractions of 1 ml were collected and assayed for enzyme activity (comp. Fig. 2).

For spectral characterization the detergent was exchanged by phenylsuperose followed by anion-exchange chromatography as follows: 2 ml of the solubilized cytochrome *c* oxidase was applied to a FPLC-phenylsuperose HR 5/5 column (Pharmacia), and preequilibrated with buffer (C) (Bis-Tris 40 mM, EDTA 1 mM, genapol X-080 0.1%, pH 6.3). The enzyme did not bind to this column and was eluted in buffer (C). Fractions with high cytochrome *c* oxidase activity were pooled and applied to a FPLC-Mono Q HR 5/5 column, preequilibrated with buffer (C). Elution was performed by a $LiClO_4$ gradient (0–0.16 M, 1 ml fractions) and fractions with cytochrome *c* oxidase activity were pooled and desalted by Pd 10 columns (Pharmacia). Absorption spectra were recorded (Shimadzu UV-300) after purification of 10 ml of solubilized enzyme. FPLC-column chromatography was performed at room temperature.

Analytical methods

Protein was determined according to [10] using the Biorad assay, chlorophyll according to [11]. Cytochrome *c* oxidase activity was measured by oxidation of reduced horse-heart cytochrome *c*-550 described in [7]. For *in vivo* measurements of oxygen uptake (Fig. 1), a Clark-type oxygen electrode was used [12], and a buffer system containing 10 mM $MgCl_2$, 6 mM Na_2HPO_4/KH_2PO_4, 10 mM HEPES, pH 7.8 [13].

Results

Ammonia induction and cyanide inhibition of respiration

After a 26 h cultivation period the cytochrome *c* oxidases activity of nitrate- and ammonia-grown *P. foveolarum* was compared (Table I). In ammonia-grown cultures the enzyme activity increased 4-fold with respect to protein and chlorophyll. The growth rate was found identical either using ammonia or nitrate as nitrogen source.

Table I. Content of cytochrome *c* oxidase in membrane material of *Phormidium foveolarum* grown in nitrate and ammonia, respectively.

Nitrogen source in growth medium	Enzyme activity $\left[\frac{\text{nmol } O_2}{\text{mg Chl} \times \text{min}}\right]$	$\left[\frac{\text{nmol } O_2}{\text{mg protein} \times \text{min}}\right]$
Nitrate	253 ± 133	166 ± 82
Ammonia	1066 ± 200	733 ± 130

After a 26-h of growth period (the inoculation was equivalent to 1 μg chlorophyll (Chl) × ml^{-1}) filaments of *Phormidium* were harvested and passed through a French-pressure cell. Enzyme activity of membrane material (without soluble proteins) was determined photometrically by cytochrome *c* oxidation and this oxidation converted stoichiometrically to oxygen uptake. Data are given as average values ± δ from 10 different batch cultures.

Oxygen uptake of intact cells was completely inhibited with 1 mM cyanide. Fig. 1 shows the inhibi-

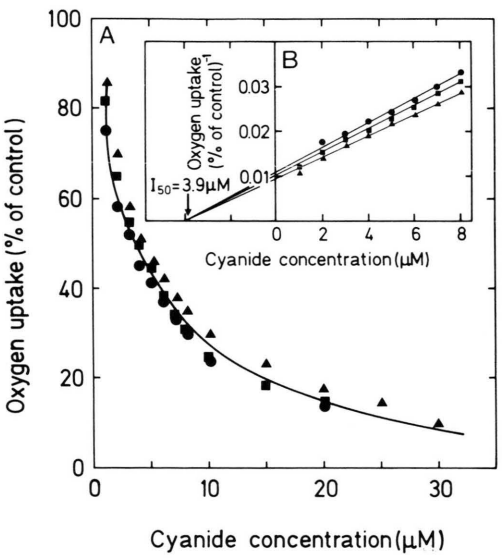

Fig. 1. Cyanide inhibition of respiration using intact filaments. (A) *Phormidium* was grown in nitrate-containing medium and harvested after 1 (▲), 2 (■) and 3 (●) days equivalent to a chlorophyll content of 5, 22, and 40 μg chlorophyll × ml^{-1}, respectively. Cyanide inhibition of oxygen uptake was determined in the oxygen electrode with intact filaments. The oxygen uptake of the control was 20–30 nmol O_2 × ml^{-1} × min^{-1}. (B) Dixon plot of respiratory cyanide inhibition of the three batch samples.

tion by cyanide of cultures grown for 1, 2 and 3 days. The inhibition was independent of the density of the culture, exhibiting an I_{50}-value of 3.9 μM.

Preparation of cytochrome c oxidase

Cytochrome *c* oxidase was solubilized by the combined application of deoxycholate (0.2% v/v) and octyl-β-D-thioglucoside (1% v/v) with a 100%-yield. The solubilized enzyme was stable for several hours at 4 °C and for several months in liquid N_2.

A typical elution pattern from gel filtration in the presence of deoxycholate is shown in Fig. 2. Enzymatic activity was found in fraction no. 6 and 7 containing only 26% of enzymatic activity loaded to the column. The recovery could be increased to 90% by adding fraction no. 10 to fractions no. 6 or 7. Fraction no. 10 itself showed no enzymatic activity. This stimulation was not possible by the addition of bovine serum albumin, phosphatidylcholine or by adding any other fraction of this gel filtration.

By detergent exchange on a phenylsuperose HR 5/5 column, 50% of contaminating carotenoids were bound to the column. During this purification step 10–20% of enzymatic activity was lost. The eluate was placed on a Mono Q HR 5/5 anion-exchange column (Fig. 3). A substantial purification was achieved by this chromatographic step due to the $LiClO_4$ gradient applied (an NaCl-gradient yielded a very poor resolution and recovery).

The specific activity increased from 33.3 nmol cytochrome *c* oxidized × mg protein^{-1} × min^{-1} of the crude cell extract to 3,580 nmol cytochrome *c* oxidized × mg protein^{-1} × min^{-1}. 80–90% of the applied enzymatic activity was recovered after chromatography on Mono Q HR 5/5. The enzyme was in the oxidized state after preparation.

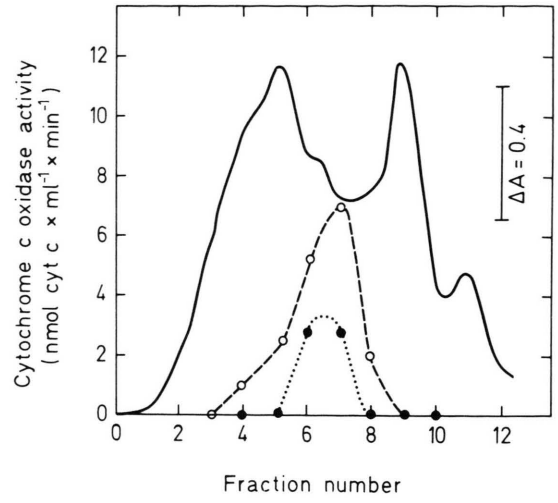

Fig. 2. Increase of activity of *Phormidium* cytochrome *c* oxidase after elution from a FPLC-Superose 6 column. The flow rate was 10 ml × h^{-1}. (——) protein content, absorbance at 280 nm, (●---●) activity of cytochrome *c* oxidase, (○– – –○) enzyme activity after addition of 20 μl of fraction no. 10 to the test tube (assay volume 1 ml).

Characterization of cytochrome c oxidase

Figure 4A and B shows determination of K_i-value for cyanide and azide. The K_i for cyanide is 0.4 μM, for azide K_i = 1.75 mM. These data agree with our studies on *Anabaena* cytochrome *c* oxidase giving a K_i-value for cyanide of 0.5 μM and $K_{i(azide)}$ of

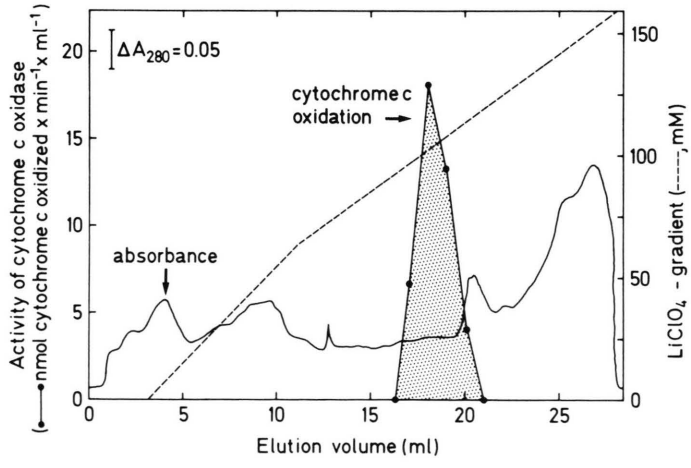

Fig. 3. Elution pattern of *Phormidium* cytochrome *c* oxidase from a FPLC-Mono Q HR 5/5 column using a $LiClO_4$-gradient. The flow rate was 2 ml × min^{-1}.

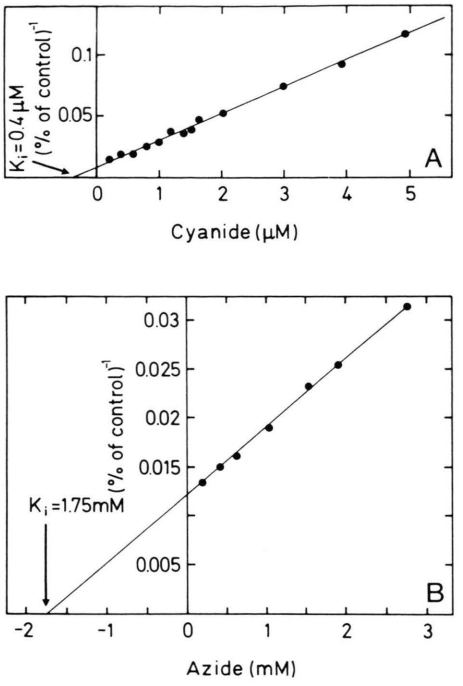

Fig. 4. Inhibition of cytochrome *c* oxidase by cyanide and azide. The activity of washed membrane material was determined photometrically. (A) inhibition with cyanide, (B) inhibition with azide.

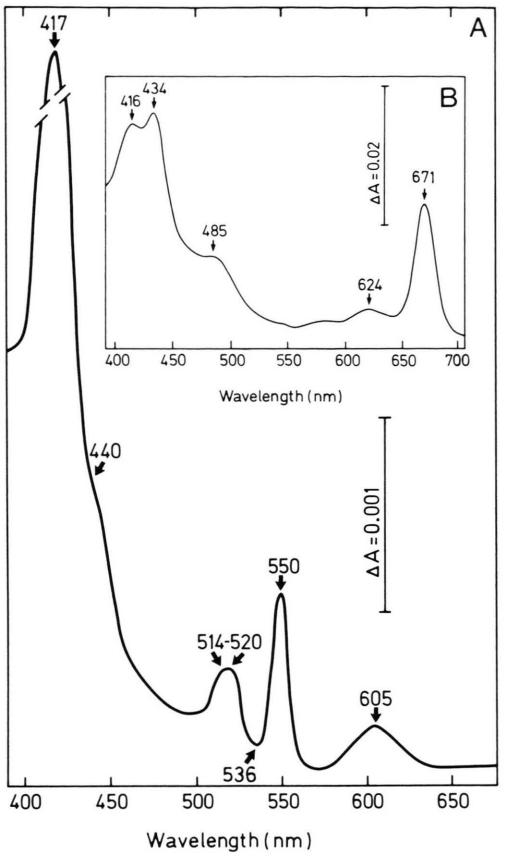

Fig. 5. (A) Difference absorption spectrum (ascorbate reduced minus oxidized) of *Phormidium* cytochrome *c* oxidase, recorded from a partially purified enzyme, with an activity of 194 nmol ferrocytochrome *c* oxidized × ml^{-1} × min^{-1}. The spectrum was corrected for the baseline. (B) Absorption spectrum from the same sample against buffer (C), see Methods.

1.25 mM. The oxygen consumption during the oxidation of ferrocytochrome *c* catalyzed by the enzyme was determined with a Clark-type oxygen electrode using limiting amounts of ferrocytochrome *c*. The molar ratio of ferrocytochrome *c* oxidized to oxygen consumed was 4.0 (data not shown). Addition of catalase to the reaction mixture had no effect. These findings clearly show that water is the reaction product of the enzymatic oxygen reduction.

The absorption spectrum (Fig. 5B) of the partial purified cytochrome *c* oxidase shows absorption peaks at 671 nm, 624 nm, 485 nm, 434 nm and 416 nm. In Fig. 5A the difference spectrum (ascorbate reduced minus oxidized as prepared) exhibits absorption peaks at 605 nm, 550 nm, 514–520 nm and 417 nm, a shoulder at 440 nm, and a trough with a sharp minimum at 536 nm.

Moreover, different purification steps – like ammonium sulfate precipitation and chromatofocusing – were performed and absorbance and difference spectra of fractions recorded containing cytochrome *c* oxidase activity. The absorption characteristics of the difference spectra of these preparations were identical to those mentioned above apart from some difference in enzyme activity (data not shown).

Discussion

A low cellular respiration rate equivalent to small amounts of cytochrome *c* oxidase together with extremely high concentrations of pigments is the main obstacle when purifying cytochrome *c* oxidase of cyanobacteria. Thus, the ammonia-induced increase of cytochrome *c* oxidase activity is a first step towards a successfull preparation. Further experiments must show whether this ammonia effect is coupled to the ammonia transport system and the protonpumping activity of the cytochrome *c* oxidase, perhaps

localized on the cytoplasmic membrane [6]. The ammoniainduced increase of respiration is due to *de novo* synthesis of the terminal oxidase, as shown by increase of the specific activity of the enzyme (Table I). Furthermore, the K_i-value towards cyanide of the terminal oxidase present in ammonia cultures is identical with the K_i of nitrate-grown cultures, both having a value of 0.4 μM. The inhibition of oxygen uptake by cyanide is characterized by one I_{50} value (Fig. 1) indicative of one terminal oxidase present [14]. Together with the lack of cyanide-insensitive respiration [15], we suggest an unbranched respiratory electron transport system with only *one* terminal oxidase present in *P. foveolarum*. An unbranched electron transport system is uncommon for bacteria [2]. Further experiments have to demonstrate whether this is true for other species or whether *Phormidium* is an exception among cyanobacteria. *Anabaena variabilis*, for instance, seems not to have a branched respiratory chain although some cyanide-insensitive oxygen uptake is observed [15].

To further characterize the enzyme it was solubilized and applied to a phenylsuperose and an anion-exchange column. The findings with the Mono Q column demonstrate the extreme hydrophobicity of the enzyme: The elution of proteins with an NaCl gradient did not produce any separation and recovery of enzymatic activity was poor (80% loss, data not shown). Due to the chaotropic effect of $LiClO_4$ [16], both good separation and recovery of the enzyme were achieved.

The partially purified cytochrome *c* oxidase is characterized by a high cyanide sensitivity (K_i = 0.4 μM) and a lower azide sensitivity (K_i = 1.75 mM), both inhibitor characteristics being indicative of cytochrome aa_3 oxidase [17].

The spectrum of the partially purified oxidase still shows contamination with chlorophyll (maxima at 671 nm and 434 nm), phycobiliproteins (maximum at 624 nm) and carotenoids (peaking at 485 nm). Apparently, the absorption at 416 nm is due to the γ-band of cytochrome aa_3. Cytochrome aa_3 is also defined by the absorption maximum at 605 nm and the shoulder at 440 nm in the difference spectrum [2]. Sharp maxima at 550 nm and at 417 nm and a broad peak at 514–520 nm are characteristic for reduced cytochrome *c*. Cytochrome *c*-550 is sometimes mixed up with a degradation product of cytochrome *f* which may be formed during preparation [18]. The marked absorbance increase in the Soret region by reduction – as can be seen in the difference spectrum of Fig. 5A – is typical of native "*c*" type cytochromes, while the Soret-band absorbance of converted cytochrome *f* does not increase by reduction [19]. A molar ratio of the cytochromes present in the preparation cannot be evaluated yet because cytochrome aa_3 is only partially reduced by ascorbate [20]. A complete reduction by dithionite failed due to precipitations. Presumably cytochrome *c* is firmly bound to the terminal oxidase prepared, which therefore may be called cytochrome caa_3 more appropriately.

We do not assume an artificial copurification of cytochrome aa_3 and cytochrome *c*-550 for the following reasons: (i) Although cytochrome *c* oxidase of *Anabaena variabilis* [7] was prepared quite similar to that of *Phormidium* described here, no cytochrome *c*-550 was present in any fraction collected in the study of the *Anabaena* oxidase. (ii) Similar spectra for cytochrome oxidase were obtained with samples after quite different purification steps like ammonium-sulfate precipitation and chromatofocusing. (iii) The results of the gel filtration experiment (Fig. 2) can be interpreted in terms of an caa_3 oxidase: gel filtration was performed in a buffer containing 0.2% deoxycholate. With ionic detergents present like deoxycholate cytochrome *c* oxidase is predominant in its monomeric form, while in nonionic detergents like octyl-β-D-thioglucoside most of the enzyme exists in the dimeric state [21]. The severe loss and subsequent recovery of enzymatic activity after gel filtration may be explained by the instability of the monomeric form which may disintegrate into a larger cytochrome aa_3 part (fractions 6 and 7) and a smaller cytochrome *c*-550 (fraction 10). Unfortunately, a spectroscopic characterization of the fractions is not yet possible due to the low enzyme content obtained and the contamination by pigments.

Although a terminal oxidase of the caa_3 type has been reported for several bacteria [22–24], this first evidence for the occurrence of such an oxidase type being present in cyanobacteria certainly deserves further substantiation by a complete purification of the intact complex.

Acknowledgements

This study was supported by Deutsche Forschungsgemeinschaft within the Special Collaborative Programme (SFB) no. 248 "Cycling of Matter in Lake Constance".

[1] P. Steinbrücke, G. C. M. Steffens, G. Panskus, B. Buse, and B. Ludwig, Eur. J. Biochem. **167,** 431–439 (1987).
[2] B. Ludwig, FEMS Microbiol. Rev. **46,** 41–56 (1987).
[3] W. J. Sweet and J. A. Peterson, J. Bacteriol. **133,** 217–224 (1978).
[4] J. M. Miller and R. B. Gennis, J. Biol. Chem. **258,** 9159–9165 (1983).
[5] G. Peschek, in: The Cyanobacteria (P. Fay and C. Van Baalen, eds.), pp. 119–161, Elsevier Science Publ., Amsterdam 1987.
[6] V. Molitor, W. Erber, and G. A. Peschek, FEBS Lett. **204,** 251–256 (1986).
[7] U. Häfele, S. Scherer, and P. Böger, Biochim. Biophys. Acta **934,** 186–190 (1988).
[8] G. A. Peschek, Biochim. Biophys. Acta **635,** 470–475 (1981).
[9] N. Wastyn, A. Achatz, M. Trnka, and G. A. Peschek, Biochem. Biophys. Res. Commun. **149,** 102–111 (1987).
[10] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[11] G. Mackinney, J. Biol. Chem. **140,** 315–322 (1941).
[12] S. Scherer and P. Böger, Arch. Microbiol. **132,** 329–332 (1982).
[13] E. Stürzl, S. Scherer, and P. Böger, Physiol. Plant. **60,** 479–483 (1984).
[14] C. Edwards, FEMS Microbiol. Lett. **21,** 319–322 (1984).
[15] S. Scherer, U. Häfele, G. H. J. Krüger, and P. Böger, Physiol. Plant. **72,** 379–384 (1988).
[16] Y. Hatefi and G. Hanstein, Methods Enzymol. **XXXI,** 770–790 (1974).
[17] R. K. Poole, Biochim. Biophys. Acta **726,** 205–243 (1983).
[18] C. Gomez-Lojero, B. Perez-Gomez, and G. Ayala, in: Proc. 5th Intern. Congr. Photosynth. (G. Akoyunoglou, ed.), **Vol. II,** pp. 599–610, Balaban International Science Services, Philadelphia 1981.
[19] J. Alam, J. Sprinkle, M. A. Hermodson, and D. W. Krogmann, Biochim. Biophys. Acta **766,** 317–321 (1984).
[20] J. P. Houchins and G. Hind, Plant Physiol. **76,** 456–460 (1984).
[21] N. C. Robinson and R. A. Capaldi, Biochemistry **25,** 2328–2335 (1977).
[22] N. Sone, T. Ohyama, and Y. Kagawa, FEBS Lett. **106,** 39–42 (1979).
[23] J. A. Fee, M. G. Choc, K. L. Findling, R. Lorence, and T. Yoshida, Proc. Natl. Acad. Sci. U.S.A. **77,** 141–151 (1980).
[24] M. Kitada and T. A. Krulwich, J. Bacteriol. **158,** 963–966 (1984).