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Study of Redox Potential in Cytochrome *c* Covalently Bound to Terminal Oxidase of Alkaliphilic *Bacillus pseudofirmus* FTU

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Abstract—Spectroelectrochemistry was used to determine the midpoint redox potentials of heme cofactors of the caa_3-type cytochrome oxidase from the alkaliphilic bacterium *Bacillus pseudofirmus* FTU. The apparent midpoint potentials (E_m^{app}) for the most prominent transitions of hemes *a* and a_3 (+193 and +334 mV, respectively) were found to be similar to the values reported for other enzymes with high homology to the caa_3-type oxidase. In contrast, the midpoint potential of the covalently bound cytochrome *c* (+89 mV) was 150-170 mV lower than in cytochromes *c*, either low molecular weight or covalently bound to the caa_3 complex in all known aerobic neutralophilic and thermo-neutralophilic bacteria. Such an unusually low redox potential of the covalently bound cytochrome *c* of the caa_3-type oxidase of alkaliphilic bacteria, together with high redox potentials of hemes *a* and a_3, ensures more than twice higher difference in redox potentials inside the respiratory complex compared to the homologous mitochondrial enzyme. The energy released during this redox transition might be stored in the transmembrane H^+ gradient even under low Δp in the alkaline environment of the bacteria at the expense of a significant increase in ΔG of the coupled redox reaction.

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Cytochrome *c* oxidase is the key enzyme of respiration and energy transformation in mitochondria and most aerobic bacteria. This terminal component of the respiratory chain is located in membranes and takes part in the consecutive transfer of electrons from respiratory substrates to oxygen. Cytochrome oxidase catalyzes transfer of four electrons and four protons to molecular oxygen, ensuring reduction of the latter into two water molecules. A part of the free energy of this thermodynamically advantageous reaction is transformed by the enzyme into electrochemical gradient of protons across the membrane ($\Delta\tilde{\mu}H^+$) during translocation of four additional protons through the membrane. Cytochrome oxidase of mitochondria, and a number of bacteria, is a mini electrical chain that contains four redox centers. The major, or, alternatively, the catalytic subunit I of these oxidases contains three redox centers, hemes *a* and a_3 and copper Cu_B. The fourth redox center (Cu_A) is located on subunit II. The X-ray 3D-structure of cytochrome oxidase has been resolved for mitochondrial [1, 2] and bacterial enzymes of *Paracoccus denitrificans* [3, 4] and *Rhodobacter sphaeroides* [5]. The mentioned enzymes are classified as the aa_3-type oxidases. A number of bacterial strains, mainly of halo-, alkali-, and thermophilic species, were shown to contain an oxidase of the caa_3-type homologous to the aa_3-type [6]. In contrast to the aa_3-type oxidase, the caa_3-type oxidase contains a covalently bound to subunit II cytochrome *c* providing one more redox center (heme *c*) in addition to Cu_A. It has been shown that even a partial deficiency in the caa_3-type oxidase as the result of mutations makes alkaliphilic bacteria incapable of living in alkaline conditions [7]. Two bacterial strains, alkaliphilic *Bacillus cognii* YN-2000 [8] and thermophilic *Bacillus* sp. PS3 [9], were shown to contain *aco*-type oxidases homologous to the caa_3-type oxidases; they also contain cytochrome *c* covalently bound to subunit II. The redox potentials of the low molecular weight cytochromes *c* in alkaliphilic bacteria and the cytochrome *c* of the only studied *aco*-type oxidase of alkaliphilic bacterium *B. cognii* YN-2000 were shown to be lower than in the water sol-

Abbreviations: OG) *n*-octyl-β-D-glucopyranoside; Δp) proton motive force; ΔG) free energy of the reaction.

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uble low molecular weight cytochrome *c* that is an electron donor for the aa_3-type cytochrome oxidase of mitochondria and neutralophilic bacteria [10]. One can speculate that the catalytic mechanism of the caa_3-type cytochrome oxidase reflects the reason for the emergence of the enzyme, that is, the necessity of proton translocation out of the membrane for effective energy conservation or, in other words, an adaptation of microorganisms to the environment.

The aim of the present study was to determine the redox properties of the covalently bound cytochrome *c* in the caa_3-type oxidase of alkaliphilic bacterium *B. pseudofirmus* FTU and (i) to elucidate the electron transfer pathway in the enzyme, (ii) to compare the redox potential of cytochrome *c* in the caa_3 cytochrome and of water soluble low molecular weight cytochromes *c*, (iii) to work out a hypothesis on the role of covalently bound cytochrome *c* in the studied enzyme.

MATERIALS AND METHODS

Isolation and purification of the enzyme. The first step of the cytochrome caa_3 isolation was the extraction of membrane proteins in the presence of detergent *n*-octyl-β-D-glucopyranoside (OG). Membrane particles were isolated as previously described [11]. Before protein extraction, the membrane particles were homogenized in the buffer for membrane isolation supplemented with KCl and phenylmethylsulfonyl fluoride (final concentrations 0.7 M and 0.17 mM, correspondingly). Then OG was added up to 2 mg per 2-5 mg of protein. Proteins were extracted over 20 min at 0°C. After that the non-solubilized particles were removed by centrifugation (20,000*g*, 20 min, 4°C), and the extracted proteins were fractionated in the obtained supernatant using ammonium sulfate. The dark-reddish floating protein fraction precipitating at 80-90% of saturated ammonium sulfate and enriched maximally in cytochrome *a* was separated from the solution by centrifugation (20,000*g*, 30 min, 4°C). The floating fraction was solubilized in 25 mM Tris-NaOH buffer (pH 7.6) containing 100 mM NaCl and 25 mM OG; the solubilized protein fraction was further used for the caa_3-type oxidase purification. A DEAE-Toyo-Pearl (Toyo-Soda, Japan) column was used for the ion-exchange chromatography. The protein mixture was fractionated by a concentration gradient of NaCl (from 200 to 500 mM) in 25 mM Tris-NaOH buffer (pH 7.6) containing 25 mM OG. Fractions with the maximum specific content of the cytochrome caa_3 were combined and concentrated at 4°C in Centricon ultraconcentrating units (Amicon, USA) of maximum working volume 2 ml. The finally obtained enzyme preparation contained 17 nmol heme *a* per mg of protein and was characterized by respiratory activity of 14 μg-atom O/min per mg protein in the presence of 0.5 mM N,N,N′,N′-tetramethyl-*p*-phenylenediamine and 5 mM ascorbate.

Spectroelectrochemistry. Spectropotentiometric redox titration of the cytochrome caa_3 was performed using an optically transparent, thin layer electrode (OTTLE) cell with a standard three-electrode configuration. Redox potentials within the range from −400 to +600 mV were set with ±20 mV step during both reductive and oxidative titration using a PAR263A potentiostat–galvanostat (Princeton Applied Research, USA). At each step, optical spectra were taken in the 350-849 nm range; spectra recorded at matching potentials during the reductive and oxidative titrations were averaged. At each potential step, the electrolysis end-point was determined by lack of changes in the cell current and optical density at 444 nm where the reduced forms of hemes *a* and a_3 of the caa_3 complex have absorption maxima (20-35 min per step). The residual cell current was typically below 50 nA. The overall potentiometric and spectral resolution of the setup was ca. ±5 mV and 0.5 nm, respectively. The experiments were performed at 21°C. The setup was PC-controlled in a fully automated mode (software Titrator 2.41 (2004-2005) designed by N. Belevich, Helsinki). All redox potentials quoted refer to the normal hydrogen electrode.

The OTTLE cell (optical pathway, 0.6 mm; volume, 15 μl) consisted of two quartz windows (diameter 20 mm) separated by a 0.25-mm thick polyether-ketone gasket with an elliptical throughcut (area, 10 mm^2) and two thinner, 50 μm Teflon gaskets served as tight-seals (the polymers were from Goodfellows Co., UK). Two gold minigrids (99.99% Au, 300 lpi, each of 70% transmittance) (Buckbee-Mears Europe GmbH, Germany) served as a working electrode. The gold surface was cleaned by repeated sonication in acetone, ethanol, and water, and then chemically modified by 12-h incubation in an aqueous solution of 2 mM cysteamine and 2 mM mercaptopropionic acid. A Pt wire immersed into 3 M KCl and a saturated Ag/AgCl half-cell served as the counter and reference electrodes, respectively. The two latter electrodes were connected to the cell by means of Vycor porous quartz-fretted liquid junctions. The connections were made through holes drilled into one of the quartz windows and glued with epoxy. In order to secure good anaerobic conditions, the OTTLE cell was placed into a vacuum/gas-tight Plexiglas box (volume, 0.6 liter) equipped with vacuum/gas valves (Swagelok) and optical windows for the spectrophotometer light beam.

To accelerate the equilibrium onset between the working electrode and molecules of the enzyme, redox mediators were added: hexaammineruthenium (E_m = +50 mV), pentaamminechlororuthenium (E_m = −130 mV), pentaamminepyridineruthenium (E_m = +250 mV), and ferrocenyl ethanol (E_m = +415 mV). Hexaammineruthenium and pentaamminechlororuthenium did not contribute to the optical density within the spectral range studied; a minor contribution from pentaamminepyridineruthenium and ferrocenyl ethanol was corrected before further data analysis.

For spectroelectrochemistry, the enzyme samples were diluted (1 : 50 v/v) in buffer A (300 mM KH_2PO_4/K_2HPO_4, KOH, pH 7.6, 25 mM OG) and concentrated twice on Centricon filters (Amicon) until final volume 15 µl. The concentration of NaCl in the final sample did not exceed 1 mM. The electrochemical cell was loaded with 12-µl mixture of the enzyme sample and mediators. Final concentration of the cytochrome *caa*$_3$ in the cell was ~50 µM.

RESULTS AND DISCUSSION

The isolated and purified cytochrome *c* oxidase of the *caa*$_3$-type was characterized using equilibrium redox titration in the UV-Vis-NIR range under strictly anaerobic conditions. Four major linearly independent spectral features were found, reflecting redox transitions of four one-electron sites of the enzyme corresponding to hemes c_{552}, c_{551}, *a*, and a_3. Figures 1 and 2 show the titration curves and the corresponding spectral components for the heme cofactors of the *caa*$_3$-type oxidase. Since hemes *a* and a_3 most probably titrated as a pair of strongly anti-cooperatively interacting sites (by analogy to the titration of hemes *a* and a_3 in the *aa*$_3$ cytochromes of mammalian mitochondria or bacterium *P. denitrificans*), determination of their individual contribution to the titration profile of the hemes meets serious problem and is impossible without inhibitor analysis (spectra in the presence of carbon monoxide or cyanide). Therefore, in the present study we had no intention to separate hemes *a* and a_3 spectrally using their titration curves; instead, we have analyzed the sum of their titration curves. The summary titration curve gave two major apparent transitions with the apparent potentials, E_m = +193 and +334 mV. As for anti-cooperative titration, both transitions were to be present in each of the putative discrete titration profiles, the above-mentioned potentials were inherent to either of the hemes *a* and a_3. Modeling the redox titration profiles for each of the hemes *a* and a_3 at 444 and 600 nm (γ and α spectral absorbance bands of the hemes) made it possible to estimate the range of contribution of each transition in the titration curve of both hemes, which were presented essentially (to 60-70%) by low potential transition (E_m = +193 mV) for heme *a* and by high-potential transition (E_m = +334 mV) for heme a_3.

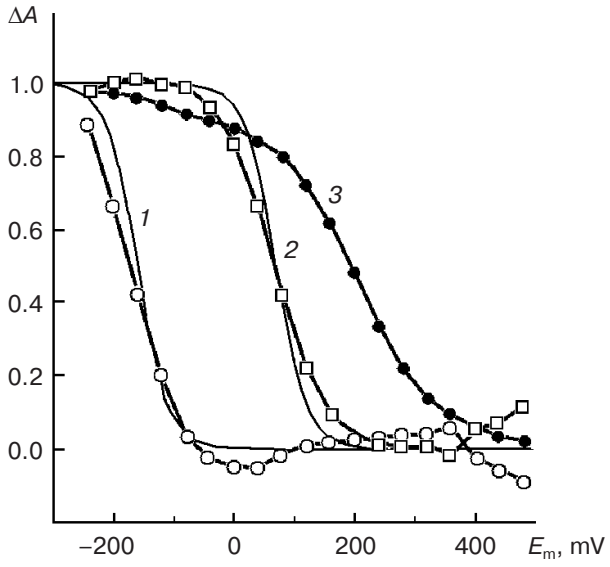

Fig. 1. Redox titration curves of the *caa*$_3$-type oxidase from *B. pseudofirmus* FTU: *1*) redox transition (E_m = −150 mV) corresponding to cytochrome c_{552}; *2*) redox transition (E_m = +89 mV) corresponding to cytochrome c_{551}; *3*) the sum of two redox transitions (E_m = +193 and +334 mV) corresponding to hemes *a* and a_3. (The small, low potential component of heme *a* and a_3 titration curve (E_m = −80 mV) might be explained by a spectral impurity produced by cytochrome *c* and was skipped for further analysis.)

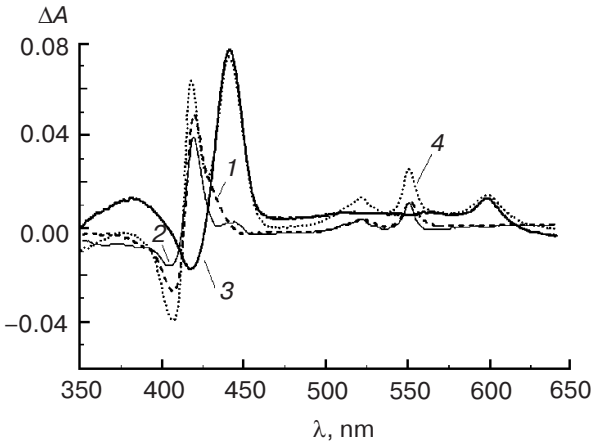

Fig. 2. Spectral components of cytochrome *caa*$_3$ from *B. pseudofirmus* FTU: *1*) cytochrome c_{552}; *2*) cytochrome c_{551}; *3*) cytochromes ($a + a_3$); *4*) the calculated sum of spectra *1* to *3*. The total redox difference (fully reduced "minus" fully oxidized) absorbance spectrum of the cytochrome *caa*$_3$ coincided with curve *4*. For the redox potentials, see Fig. 1.

The redox titration curves for both cytochromes *c* appeared slightly less steep than expected for a simple, single-electron titration profile. At the same time, the amplitude ratio of the redox difference spectra revealed that the two cytochromes *c* were in equimolar ratio as related to heme content (1 : 1). Interestingly, the cytochrome c_{552} co-purified with the *caa*$_3$ complex was characterized by a very low redox potential (−150 mV). This is the lowest redox potential value of the known cytochromes *c* of aerobic bacteria; lower redox potentials are known only for multiheme cytochromes *c* of anaerobic bacteria [12]. We suggest that the cytochrome c_{552} is the native membrane-bound electron donor to the *caa*$_3$ complex; indeed, the enzyme activity was maximal in its presence and decreased, on average, by 2-3 times upon its separation from the complex in the presence of 30 mM

OG. Such a loss of the activity was reversible and could be restored by the addition of cytochrome c_{552} to the enzyme. In a separate set of experiments with the caa_3 complex upon separation of cytochrome c_{552} from it in the presence of 30 mM OG, we demonstrated that the redox component with the midpoint potential of –150 mV disappeared from the titration profile (data not shown). According to our data, the covalently bound cytochrome c_{551} has the apparent midpoint potential of +89 mV. The potential value of the cytochrome c_{551} determined in this study is 150-170 mV lower than for cytochromes *c* of the known aerobic neutralophilic and thermo-neutralophilic bacteria, either covalently bound to the caa_3 complex or not included in the complex. The only one example where cytochrome *c* is an integral part of the *aco*-type oxidase of alkaliphilic bacterium *B. cognii* YN-2000 [13] shows a similarly low redox potential, +95 mV (see table). The apparent midpoint potential of hemes *a* and a_3 of *B. pseudofirmus* FTU is in the same range of values as those of the known neutralophilic and thermo-neutralophilic bacteria: *Bacillus* sp. PS3, *Thermus thermophilus*, *B. stearothermophilus*, *B. subtilis*, *Heliobacterium gestii*, and *P. denitrificans*.

The table presents the redox potentials of heme-containing prosthetic groups of the known oxidases with covalently bound cytochrome *c*. Up to now such oxidases were presented by the caa_3 and *aco* cytochrome complexes that have been found in three groups of bacteria different in their environmental conditions: in alkaliphiles, thermo-neutralophiles, and neutralophiles. The table shows that the covalently bound cytochrome *c* of oxidase complexes of neutralophilic bacteria is high-potential (E_m = +250 mV), which is similar to the feature of the soluble cytochrome *c* serving as an electron donor for the well studied mitochondrial and bacterial aa_3-type oxidases. The sharp deviation of redox potential in the low potential area can be seen in covalently bound cytochrome *c* of alkaliphilic bacteria. Note that in this study we have obtained such data for the first time for the caa_3 complex in alkaliphilic bacteria. It is worth mentioning that on energy coupling type the caa_3-type oxidase of *B. pseudofirmus* FTU ranks with the aa_3-type oxidases. The purified enzyme reconstituted in azolectin liposomes displays proton pump features with the $H^+/e^- = 1$ in the presence of substrates [14].

Our study opens a possibility to make a comparison inside the oxidase group of the same heme composition (the caa_3 complex) and to reveal the influence of alkalinity of the environment on cytochrome oxidase features. Here we showed that, indeed, the covalently bound cytochrome *c* of the caa_3-type oxidase from alkaliphilic bacteria is characterized by an unusually low redox potential, which could be of functional importance for the enzyme. We suggest here a new model for a design of the proton pump (such as the caa_3-type oxidase) in alkaliphilic bacteria. These enzymes are provided with the particular "entry gates" for an electron transmitted to the enzyme from the upper points of respiratory chain. The

Dependence of redox potential of heme cofactors on the relation of bacteria to ecological niche

Bacterial strain or animal organ	Relation of the strain to alkalinity of the medium	Cytochrome	Midpoint redox potential of heme cofactor, mV		
			c	*a*	a_3
B. cognii YN-2000 [13]	alkaliphile	*aco*	+95	+323	+250
B. pseudofirmus FTU	»	caa_3, c_{551} c_{552}	+89 –150	+193	+334
B. alcalophilus [15]	»	caa_3	–	+230	+390
Rhodothermus marinus [16]	thermo-neutralophile	caa_3, c_{552}	+260	+255	+180
Bacillus sp. PS3 [17]	»	caa_3, c_{551}	+225	+340	+190
Thermus thermophilus [18]	»	caa_3	+218	+378	+133
B. subtilis [19]	neutralophile	caa_3	–	–	–
P. denitrificans	»	aa_3		+360	+220
Bovine heart		aa_3		+365	+210
Horse heart		soluble c_{550}	+250		

Note: Sign "–" means the absence of data in literature.

"entry gates" are presented by an unusually low potential redox group of cytochrome *c*. The potential difference between the cytochrome *c* and heme a_3 in the caa_3 cytochrome is 0.24 V, which is more than twice higher than the same value in the caa_3 and aa_3 cytochromes of neutralophilic bacteria and mitochondria (ca. 0.11 V). With the relatively high positive value of the heme *a* redox potentials, such a feature of the covalently bound cytochrome *c* in the caa_3-type oxidase from alkaliphilic bacteria might serve as a mechanism ensuring energy conservation in proton gradient on the membrane even under low Δp in alkaline environment at the expense of a considerable increase of the ΔG absolute value of the coupled redox reaction.

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REFERENCES

1. Tsukihara, T., Aoyama, H., Yamashita, E., Takashi, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science*, **272**, 1136-1144.
2. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) *Science*, **280**, 1723-1729.
3. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature*, **376**, 660-669.
4. Ostermeier, C., Harrenga, A., Ermler, U., and Michel, H. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 10547-10553.
5. Svensson-Ek, M., Abramson, J., Larsson, G., Tornroth, S., Brzezinski, P., and Iwata, S. (2002) *J. Mol. Biol.*, **321**, 329-339.
6. Pereira, M. M., Santana, M., and Teixeira, M. (2001) *Biochim. Biophys. Acta*, **1505**, 185-208.
7. Krulwich, T. A., Ito, M., Gilmour, R., Sturr, M. G., Guffanti, A. A., and Hicks, D. B. (1996) *Biochim. Biophys. Acta*, **1275**, 21-26.
8. Sone, N., Ogura, T., Noguchi, S., and Kitagawa, T. (1994) *Biochemistry*, **33**, 849-855.
9. Yumoto, I., Takahashi, S., Kitagawa, T., Fukumori, Y., and Yamanaka, T. (1993) *J. Biochem.*, **114**, 88-95.
10. Yumoto, I. (2002) *J. Biosci. Bioeng.*, **93**, 342-353.
11. Muntyan, M. S., and Skripnikova, E. V. (1993) *Biochemistry (Moscow)*, **58**, 928-933.
12. Moura, J. J. G., Costa, C., Liu, M.-Y., Moura, I., and LeGall, J. (1991) *Biochim. Biophys. Acta*, **1058**, 61-66.
13. Orii, Y., Yumoto, I., Fukumori, Y., and Yamanaka, T. (1991) *J. Biol. Chem.*, **266**, 14310-14316.
14. Grischuk, Y. V., Muntyan, M. S., Popova, I. V., and Sorokin, D. Y. (2003) *Biochemistry (Moscow)*, **68**, 385-390.
15. Lewis, R. J., Prince, R. C., Dutton, P. L., Knaff, D. B., and Krulwich, T. A. (1981) *J. Biol. Chem.*, **256**, 10543-10549.
16. Pereira, M. M., Carita, J. N., and Teixeira, M. (1999) *Biochemistry*, **38**, 1268-1275.
17. Poole, R. K. (1983) *Biochim. Biophys. Acta*, **726**, 205-243.
18. Hellwig, P., Soulimane, T., and Mantele, W. (2002) *Eur. J. Biochem.*, **269**, 4830-4838.
19. Hicks, D. B., and Krulwich, T. A. (1995) *Biochim. Biophys. Acta*, **1229**, 303-314.