Biochimica et Biophysica Acta, 1143 (1993) 142–146

BBABIO 43843

Two types of terminal oxidase in alkalotolerant *Bacillus FTU*

M.S. Muntyan and E.V. Skripnikova

A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow (Russia)

(Received 15 October 1992)
(Revised manuscript received 26 January 1993)

Key words: Terminal oxidase; Oxidase; Cytochrome *a*; Cytochrome *o*; Alkalotolerant bacterium; (*Bacillus FTU*)

The terminal segment of the *Bacillus FTU* respiratory chain has been studied. The shape of difference spectra and biphasic cyanide titration curve point to the presence of two terminal oxidases (aa_3- and *o*-type) in the subcellular membrane vesicles of this *Bacillus*. The aa_3-type oxidase was shown to dominate in the beginning of the logarithmic phase while the *o*-type oxidase was more pronounced at the stationary phase of bacterial growth. Both enzymes oxidized TMPD in the presence of ascorbate: the *o*-type oxidase with $K_m = 0.7$ mM TMPD and $V_{max} = 2.2$ μmol O_2 min^{-1} $nmol^{-1}$ haem *o* and the aa_3-type oxidase with $K_m = 2$ mM TMPD and $V_{max} = 11.2$ μmol O_2 min^{-1} $nmol^{-1}$ haem *a*. KCN inhibited the two oxidases (half-maximal inhibition at 2 μM and 20 μM for the aa_3- and *o*-type oxidases, respectively).

Introduction

Bacillus FTU is an alkalo- and halotolerant bacterium that grows well within the pH interval of 8.0–9.0 [1]. The subcellular *Bac.FTU* vesicles were shown to be competent in Na^+ and H^+ transport coupled to ascorbate oxidation via TMPD [1,2]. Micromolar cyanide concentrations inhibited the H^+ transport. Much higher concentrations of the inhibitor were required to suppress the Na^+ transport [3].

It has not been shown yet what kinds of oxidase are inherent in *Bac.FTU* and which of them might take part in the described Na^+ transport. It is well known that members of Genus *Bacillus*, like many other procaryotes, possess a branched respiratory chain with multiple terminal oxidases. The presence of aa_3-, caa_3-, *o*-, *aco*- and *d*-type oxidases in the membranes of *Bacillus* has been described [4–8].

In the present paper we have shown that *Bac.FTU* membranes contain at least two kinds of terminal oxidase, namely the aa_3-and *o*-types. The former and the latter oxidases proved to be typical for the logarithmic and stationary phases of bacterial growth.

Correspondence to: M.S. Muntyan, A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMSF, phenylmethylsulfonyl fluoride.

Materials and Methods

Strain and growth conditions

Alkalo- and halotolerant *Bacillus FTU* was isolated in our laboratory [1,2]. The strain was maintained by a monthly transfer on solid medium of the same content as the growth medium containing 2% agar above. For a long period the strain was preserved by lyophilization. The growth medium contained 0.5 M NaCl, 10 mM KCl, 15 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 5 mM $MgSO_4$, 10^{-5} M $FeSO_4$, 0.1 mM EDTA, 50 mM Tris-HCl (pH 8.6). 60 mM sodium succinate was used as the only energy and carbon source. Bacteria were grown aerobically in 0.7 l vessels at 200 rpm, 37°C and constant pH. The growth rate of culture in liquid medium was monitored using light scattering at 500 nm. Upon reaching the appropriate phase of growth, the cells were immediately harvested by centrifugation (10000 $\times g$, 10 min^{-1}, 4°C) and washed twice with the medium, containing 0.5 M NaCl, 5 mM Tris-HCl (pH 8.2). The washed cells were used immediately for isolation of membrane vesicles.

Isolation of membrane vesicles

The following procedures were carried out at 4°C as described in paper [2] with slight modifications. The washed cells were resuspended (0.1–0.15 g wet weight/ml) in the medium, containing 10 mM Tris-HCl (pH 8.2), 0.3 M KCl, 10 mM NaCl, 30 mM $MgSO_4$, 1 mM PMSF and lysozyme (2 mg/mg wet weight of biomass). Then the suspension was incubated for 30–40

min in a shaker at 37°C for digestion of the cell walls. The resulting spheroplasts were centrifuged (10 000 × *g*, 10 min, 4°C) and resuspended in the ice-cold medium D, containing 50 mM Tricine (pH 8.2), 0.1 M K_2SO_4, 10 mM $MgSO_4$, 2.5 mM Na_2SO_4, 0.5 mM EDTA, 1 mM PMSF, DNAase I (20 μg/ml). The cell suspension was passed two or three times through a French press cell at 700 kg/cm^2. Unbroken spheroplasts and the cell debris were removed by centrifugation (10 000 × *g*, 10 min, 4°C). The membrane vesicles were sedimented at 200 000 × *g* for 2 h, washed twice in medium D and resuspended to 20 mg/ml in medium D, containing 25% glycerol (v/w) and stored in liquid N_2.

Spectroscopic measurements

The membrane vesicle samples were dissolved in medium D and the difference spectra were recorded at room temperature, using a Hitachi U-3400 spectrophotometer. The O_2-oxidized cytochromes of membrane vesicles were taken as a baseline for the reduced-minus-oxidized difference spectrum, after that a few grains of solid sodium dithionite were added to reduce the cytochromes and the difference spectrum was recorded. Ferricyanide oxidation of the cytochromes taken as a baseline did not increase the amplitude of the peaks and was therefore omitted. To obtain the CO_2-reduced-minus-reduced difference spectrum, the air was removed from the cuvette by bubbling argon for 10 min and dithionite-reduced cytochromes were taken as a baseline. Then CO was bubbled through the cuvette for 3 min and the difference spectrum was recorded. The contents of cytochromes *a*, *b* + *o* and *c* were determined from the reduced-minus-oxidized difference spectra by using the millimolar extinction coefficient of 20.5 at 601–620 nm [9], 17.5 at 560–575 nm [10] and 17.3 at 553–537 nm [11]. The cytochrome *o* content was determined from the CO-reduced-minus-reduced difference spectra. The millimolar extinction coefficient was that used for studies of *E. coli* cytochrome *o*: 145 at 415–432 nm [12].

Other procedures

Oxygen uptake in the membrane vesicles was measured with a standard oxygen Clark-type electrode at 25°C. Protein was determined by the method of Lowry et al. [13] with BSA as a standard.

Results

The isolated *Bacillus FTU* membranes contain cytochromes *a*, *b*, *o* and *c*. The reduced-minus-oxidized difference spectra (Fig. 1) show the peak at 601 nm and the shoulder at 443.5 nm characteristic of the *a*-type cytochromes which indicate the presence of the aa_3-type oxidase. The peaks at 553 and 523 nm are typical for the α- and β-bands of the *c*-type cytochromes. The shoulders at 560 and 530 nm show the α- and β-bands of the (*b* + *o*)-type cytochromes. As one can see from Fig. 1, oxidase of the aa_3 type disappears during the growth of bacteria, decreasing from 0.37 nmol of haem *a* per mg protein to very low concentrations (lesser than 0.03 nmol of haem *a* per mg protein). An increase and then a small decrease in the (*b* + *o*)-type cytochromes content (rise and fall of the shoulders at 530 and 560 nm) are also seen in Fig. 1 and Table I, while the cytochrome *c* content (the peaks at 523 and 553 nm) increases (Table I) during the cell growth. The CO-reduced-minus-reduced difference spectra show almost the complete disappearance of the troughs at 444 and 607 nm and of the peaks at 428 and 588 nm typical for the aa_3-type oxidase, and the appearance of the troughs at 432 and 561 nm and of the peaks at 415 and 577 nm typical for the *o*-type oxidase (Fig. 2). Obviously, the trough at 555–565 nm (Fig. 2A) is heterogenic and a few *b*-type cytochromes react with CO. The *c*-type cytochrome which trough is seen at 523 nm (Fig. 2A) reacts with CO as the *a*- and the (*b* + *o*)-type cytochromes do. In contrast to the increase in the amount of cytochromes *c* seen in the reduced-minus- oxidized difference spectra, the CO-

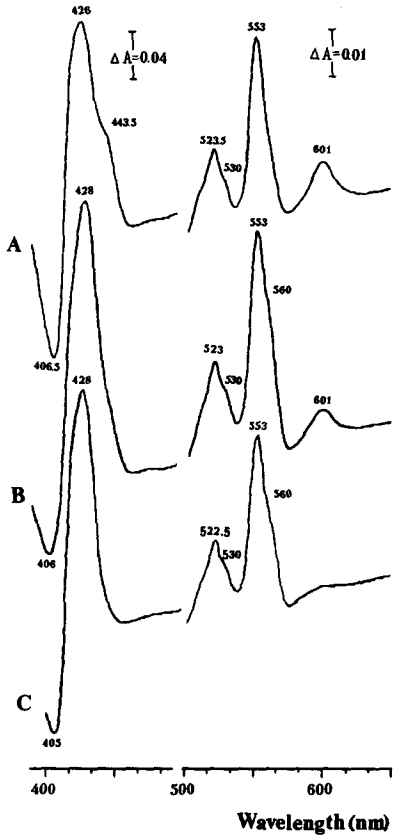

Fig. 1. Reduced-minus-oxidized difference spectra of the membrane vesicles isolated from *Bac.FTU* cells harvested in the beginning (A) and at the end (B) of the logarithmic phase or at the stationary phase (C) of growth. The protein concentrations were 1.3 mg/ml in (A), 0.8 mg/ml in (B) and (C).

TABLE I

Dependence of cytochrome contents and oxidation rate of the membrane vesicles on the growth phase of bacteria

Cytochrome	Wavelength pair (nm)	Content (nmol/mg protein)		
		beginning log	end log	stationary
c	553–537	2.2	2.5	2.7
b + *o*	560–575	1.38	2.2	1.9
o	415–432	n.d. [a]	0.17	0.25
a + a_3	601–620	0.37	0.2	n.d. [a]
V_{max} [b]		5.0 [c]		0.59 [c]
		13.9 [d]		2.4 [e]

[a] n.d., not determined.
[b] V_{max} of oxidase activity at 25°C. 10 mM ascorbate-Tris with TMPD is used as a substrate and the oxidation rate is expressed in
[c] μmol O_2 min^{-1} mg^{-1} protein;
[d] μmol O_2 min^{-1} $nmol^{-1}$ haem *a*;
[e] μmol O_2 min^{-1} $nmol^{-1}$ haem *o*.
The incubation mixture was the same as in Fig. 3.

binding *c*-type cytochrome disappears like the aa_3-type oxidase as the cells move on to the stationary phase.

It is clear from Table I and Fig. 3 that membranes from the stationary culture still display respiratory activity with TMPD and ascorbate (V_{max} = 0.59 μmol O_2 min^{-1} mg^{-1} protein) although it is nine times lower

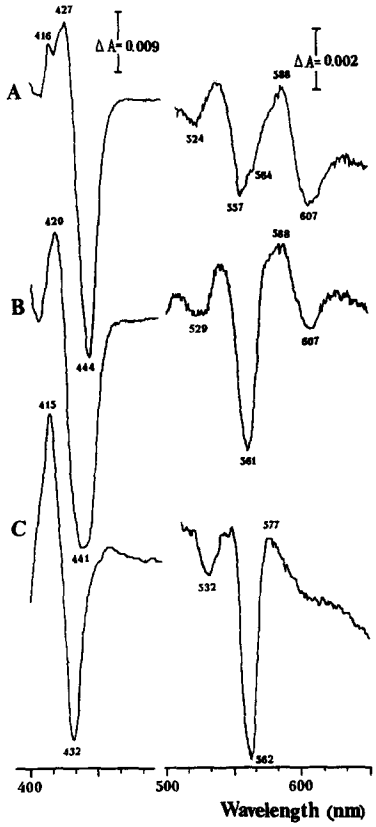

Fig. 2. CO-reduced-minus-reduced difference spectra of membrane vesicles (A, B and C were the same as in Fig. 1). The protein concentrations were 1.3 mg/ml in (A)–(C).

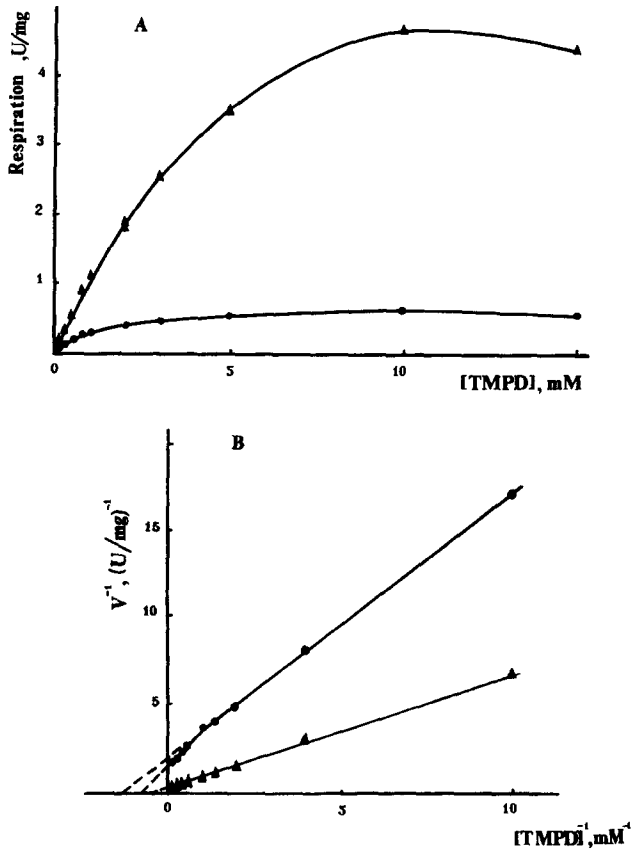

Fig. 3. Dependence of respiratory activity (U = unit is defined as the amount of enzyme preparation that oxidizes 1 μmol O_2/min) of membrane vesicles on the TMPD concentration. Membrane vesicles were obtained from *Bac.FTU* cells harvested in the beginning of the logarithmic phase (▲) and at the stationary phase (●) of growth. The incubation mixture contained medium D supplied with 10 μM ascorbate-Tris and 0.1–10 μM TMPD. Respiration was initiated by vesicles addition: 0.1 (▲) or 0.11 (●) mg protein ml.

than in the beginning of the logarithmic stage (V_{max} = 5 μmol O_2 min^{-1} mg^{-1} protein). According to the spectroscopic data (Figs. 1, 2; Table I), the more-extensively TMPD-oxidizing membrane vesicles contain mainly the aa_3-type oxidase whereas the vesicles poorly reacting with TMPD contain predominantly the *o*-type oxidase. Hence, the aa_3-type oxidase oxidizes TMPD with K_m = 2 mM while the *o*-type oxidase – with K_m = 0.7 mM (Fig. 3B). The aa_3-type oxidase appears to be more sensitive to KCN, half maximal inhibition being caused by 2 μM ($C_{1/2}$) of cyanide. The *o*-type oxidase has one order higher resistance to KCN ($c_{1/2}$ = 20 μM, Fig. 4). Additionally, Fig. 4 reveals the presence of the *o*-type oxidase in membranes obtained from the cells in the beginning of the logarithmic phase. This information cannot be obtained from Fig. 3B because of a little contribution of the *o*-type oxidase to the total oxidative activity of the membrane vesicles at saturating concentrations of TMPD in the beginning of the logarithmic phase of growth. Taking into account these relationships, it may be concluded

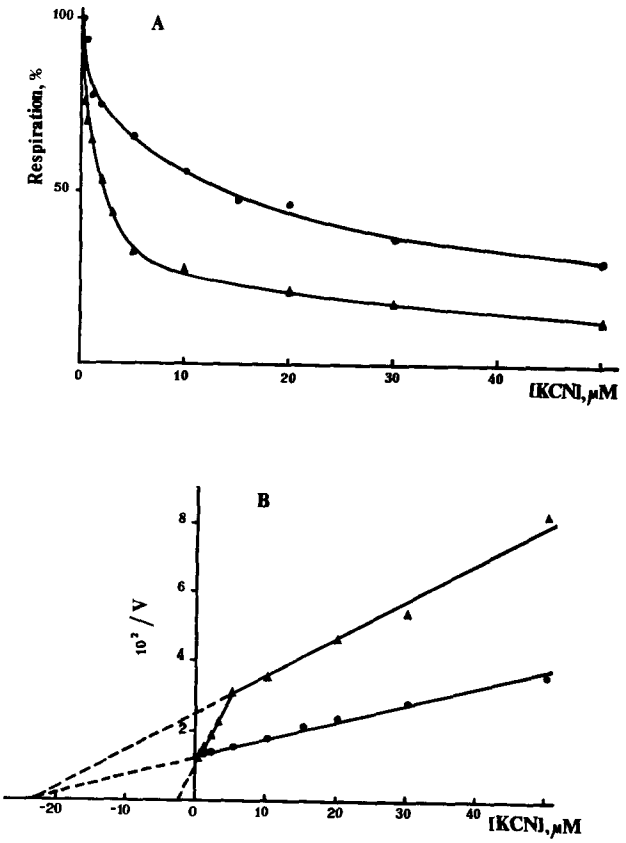

Fig. 4. Cyanide inhibition of respiratory activity of membrane vesicles. Vesicles were obtained from *Bac.FTU* cells harvested in the beginning of the logarithmic phase (▲) and at the stationary phase (●) of growth. The incubation mixture contained medium D supplied with 10 μM ascorbate-Tris, 0.05 mg protein ml (▲) or 0.11 mg protein ml (●) of membrane vesicles and no or 0.1–50 μM KCN. Respiration was initiated by TMPD addition to obtain 1 μM final concentration. 100% of respiratory activity correspond to 1.14 (▲) and 0.25 (●) μmol O_2 min^{-1} mg^{-1} protein.

that the relative contribution of the aa_3-type oxidase V_{max} to the total respiratory activity of membranes appears to be 80–85% (10.8–11.5 μmol O_2 min^{-1} $nmol^{-1}$ haem *a*) in the beginning of the logarithmic phase and it decreases to 5–10% at the stationary phase. On the contrary, the *o*-type oxidase contributes, under the same measurement conditions, 15–20% of the activity in the beginning of the logarithmic phase and 90–95% (2.1–2.3 μmol O_2 min^{-1} $nmol^{-1}$ haem *o*) of such at the stationary phase.

Discussion

Our results indicate the presence of *o*-type and aa_3-type oxidases in *Bac.FTU* membranes. The content of aa_3-type oxidase and CO-binding cytochrome *c* appears to decrease simultaneously, while that of the *o*-type oxidase and the total cytochromes *c* increases, during the growth of bacteria toward the stationary phase. Apparently, at the logarithmic phase of growth, the caa_3 complex is present in *Bac.FTU* membranes, similarly to *Bacillus cereus* [5], *Bacillus subtilis* [14], *PS 3* [15], *Bacillus stearothermophilus* [16], *Bacillus firmus RAB* [17], *Bacillus firmus OF4* [18] and *Thermus thermophilus* [19]. The possible existence of a caa_3 complex means that the content of other cytochromes *c* might increase even more strongly during the bacterial growth toward stationary phase than can be seen from the total cytochrome *c* content recorded in Table I. An increase in these other cytochromes *c* correlates with the *o*-type oxidase increase in membranes during the growth of bacteria, but the role of these cytochromes *c* is unclear.

The fact of induction of alternative oxidases during the growth of bacterial cultures is well described for *E. coli* [20,21]. In this case, the *o*-type oxidase dominated in the beginning of the logarithmic phase while the *d*-type oxidase was induced later. Hence, physiologically, the *Bac.FTU* caa_3- and *o*-type oxidases should be analogous to the *E. coli o*- and *d*-type oxidases, respectively.

Induction of a third, *d*-type oxidase in membranes of *Bacillus firmus OF4* in addition to existing oxidases of the caa_3-and *o*-type has recently been described by Hicks and co-workers [6]. The *d*-type cytochrome has not been detected in any *Bac.FTU* growth phase under our conditions.

The *Bac.FTU* vesicles were shown to be capable of Na^+- and H^+-transport when TMPD and ascorbate were used as electron donors [1,2]. It seems as if the H^+ transport described by Kostyrko et al. [3] is carried out by the aa_3-type oxidase described in our paper because both the H^+ -transport and the aa_3-type oxidase activity are inhibited by KCN with $c_{1/2} = 2$ μM. At the same time, the nature of the Na^+ transport is difficult to clarify at this moment because only 20 μM of KCN are sufficient for 50% inhibition of the *o*-type oxidase in our experiments while the Na^+ transport is inhibited by 50% with 100 μM of KCN [3]. Further investigations of this problem are in progress.

Acknowledgements

The authors wish to express their thanks to Professor V.P. Skulachev for his helpful advice and a critical review of the manuscript, and to I.Y. Fokina for assistance.

References

1 Semeykina, A.L., Skulachev, V.P., Verkhovskaya, M.L., Bulygina, E.S. and Chumakov, K.M. (1989) Eur. J. Biochem. 183, 671–678.
2 Verkhovskaya, M.L., Semeykina, A.L. and Skulachev, V.P. (1988) Dokl. Acad. Nauk SSSR 303, 1501–1503.
3 Kostyrko, V.A., Semeykina, A.L., Skulachev, V.P., Smirnova, I.A., Vagina, M.L. and Verkhovskaya, M.L. (1991) Eur. J. Biochem. 198, 527–534.

4 Edwards, C., Beer, S, Siviram, A. and Chance, B. (1981) FEBS Lett. 128, 205–207.
5 Garcia-Horsman, J.A., Barquera, B. and Escamilla, J.E. (1991) Eur. J. Biochem. 199, 761–768.
6 Hicks, D.B., Plass, R.J. and Quirk, P.G. (1991) J. Bacteriol. 173, 5010–5016.
7 Qureshi, M.H., Yumoto, I., Fujiwara, T., Fukumori, Y. and Yamanaka, T. (1990) J. Biochem. 107, 480–485.
8 Sone, N., Kagawa, Y. and Orii, Y. (1983) J. Biochem. 93, 1329–1336.
9 Van Gelder, B.F. (1966) Biochim. Biophys. Acta 118, 36–46.
10 Deeb, S.S. and Hager, L.P. (1964) J. Biol. Chem. 239, 1024–1031.
11 Jones, C.W. and Readfearm, E.R. (1966) Biochim. Biophys. Acta 113, 467–481.
12 Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259, 3368–3374.
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, A.J. (1951) J. Biol. Chem. 193, 265–275.
14 Lauraeus, M., Haltia, T., Saraste, M. and Wikstrom, M. (1991) Eur. J. Biochem. 197, 699–705.
15 Sone, N. and Yanagita, Y. (1982) Biochim. Biophys. Acta 628, 216–222.
16 de Vrij, W., Heyne, R.I.R. and Konings, W.N. (1989) Eur. J. Biochem. 178, 763–770.
17 Kitada, M. and Krulwich, T.A. (1984) J. Bacteriol. 158, 963–966.
18 Guffanti, A.A., Finkelthal, O., Hicks, D.B., Falk, L., Sidhu, A. and Krulwich, T.A. (1986) J. Bacteriol. 167, 766–773.
19 Buse, G., Hensel, S. and Fee, J.A. (1989) Eur. J. Biochem. 181, 261–268.
20 Anraku, Y. and Gennis, R.B. (1987) Trends Biochem. Sci. 12, 262–266.
21 Ingledew, W.J and Poole, R.K. (1984) Microbiol. Rev. 48, 222–271.