Biochemistry (Moscow), Vol. 69, No. 10, 2004, pp. 1165-1169. Translated from Biokhimiya, Vol. 69, No. 10, 2004, pp. 1431-1436.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-134, August 29, 2004.

Uncoupling Effect of Fatty Acids in Halo- and Alkalotolerant Bacterium *Bacillus pseudofirmus* FTU

I. V. Popova, M. E. Bodrova, E. N. Mokhova, and M. S. Muntyan*

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119992, Russia; fax: (7-095) 939-3181; E-mail: muntyan@genebee.msu.su

Received May 31, 2004

Abstract—Natural uncouplers of oxidative phosphorylation, long-chain non-esterified fatty acids, cause uncoupling in the alkalo- and halotolerant bacterium *Bacillus pseudofirmus* FTU. The uncoupling effect in the bacterial cells was manifested as decrease of membrane potential and increase of respiratory activity. The membrane potential decrease was detected only in bacterial cells exhausted by their endogenous substrates. In proteoliposomes containing reconstituted bacterial cytochrome *c* oxidase, fatty acids caused a "mild" uncoupling effect by reducing membrane potential only at low rate of membrane potential generation. "Free respiration" induced by the "mild" uncouplers, the fatty acids, can be considered as possible mechanism responsible for adaptation of the bacteria to a constantly changed environment.

Key words: fatty acids, bacteria, uncouplers, proteoliposomes, membrane potential, caa_3 type oxidase

According to the concept of non-coupling oxidation ("free" respiration), several pathways of electron transfer exist in biological membranes [1]. Besides the principal "accumulating" pathway of oxidative phosphorylation, they include energy dissipating pathways of electron transfer in redox chains that are not coupled to phosphorylation. Such type of biological oxidation was defined as non-coupling (or free) oxidation. Non-coupling leading to uncoupling of oxidative phosphorylation may be induced by either protonophore/sodiophore uncouplers or electron carriers bypassing the principal (coupled) respiratory chain. Non-phosphorylating oxidations play diverse functions within the cell. For example, it includes hepatic microsomal and bacterial membrane cytochrome P450 dependent detoxifications, burning of under-oxidized products in yeast, bacteria, and vertebrates, increased heat production in animals under cooling and in plants at certain physiological periods [2], protection against reactive oxygen species, and (surprisingly!) acceleration of ATP synthesis [3].

Uncoupling of oxidative phosphorylation by natural compounds was discovered in 1956 [4]. This phenomenon was the best studied using mitochondria isolated from homoiothermic animals. Under conditions of low ambient temperature, excess of oxidizing substrate, long chain free fatty acids, is the physiological uncoupling factor acting as protonophore [5, 6]. The protonophore effect of fatty acids on mitochondria makes substantial contribution to immediate heat production in animals under low ambient temperatures. In the present study, we have investigated the effect of fatty acids on energy coupling in alkalo- and halotolerant bacteria *Bacillus pseudofirmus* FTU. Such effect of fatty acids has not yet been investigated in prokaryotes.

MATERIALS AND METHODS

The strain of *Bacillus pseudofirmus* FTU was isolated and characterized in [7]. This strain included into the DSMZ International Collection of Microorganisms (Braunschweig, Germany) is maintained at the Belozersky Institute of Physico-Chemical Biology, Moscow State University. Lauric, myristic, and palmitic acids, azolectin, MOPS, and *n*-octyl-β-D-glucopyranoside were purchased from Sigma (USA). Fatty acids dissolved in bidistilled ethanol were used as 20 mM solutions.

Abbreviations: CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; DCCD) N,N′-dicyclohexylcarbodiimide; DNP) 2,4-dinitrophenol; HQNO) 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine; TPP^+) tetraphenylphosphonium chloride.

* To whom correspondence should be addressed.

0006-2979/04/6910-1165 ©2004 MAIK "Nauka / Interperiodica"

Bacteria cultivation. Bacteria were grown in the following mineral medium (g/liter): NaCl, 30; KCl, 0.75; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 1.23; Tris-HCl (pH 8.7), 6; EDTA, 0.03; $FeSO_4 \cdot 7H_2O$, 0.0015; KH_2PO_4, 0.28; succinate (NaOH), 5.9. All the components (except phosphates) were autoclaved together.

Preparation of cell suspension for experiments. *Bacillus pseudofirmus* FTU cells were sedimented by centrifugation at 9000*g* for 10 min and washed three times in the following buffer solution: 150 mM KCl, 450 mM NaCl, 5 mM Tris-HCl (pH 8.2). The final volume of the cell suspension was 1% of initial volume of the grown cell culture. The washed cell suspension was incubated at 4°C for 2 days to exhaust endogenous substrates; after that cells were sedimented and resuspended in the same washing medium but without the buffer component. In this medium, cells were incubated for 3 h.

Respiratory activity of cells was measured at 25°C using a standard Clark-type platinum electrode and LP7e polarograph.

Membrane potential of intact cells was measured at 25°C using a TPP^+-selective electrode [8] and a thermostatted cell, which contained 2 ml of the medium for cell washing. Measurements were carried out under constant stirring and intensive aeration. The electrode was calibrated by additions of TPP^+; the final concentration of TPP^+ was 1.4 μM. Cell suspension was added to the final density corresponding to 4.5-6.0 optical absorbance units at 500 nm.

Membrane potential of proteoliposomes was registered using an Aminco DW-2000 spectrophotometer (dual wavelength mode, $\Delta\lambda$ = 555-523) by measuring safranine O permeable cation concentrations inside and outside proteoliposomes [9]. All experiments were carried out using in a glass cuvette (10 × 10 mm); the working volume was 1 ml. A magnetic stirrer was used for aeration.

Proteoliposomes with reconstituted caa_3 type cytochrome *c* oxidase from *Bacillus pseudofirmus* FTU were obtained as described earlier [10].

Proton release from *Bacillus pseudofirmus* FTU cells was studied using the method of small oxygen additions into anaerobic suspension. Changes in pH of the reaction mixture were registered using an I-130 ionometer (Gomel, Belarus) and a combined pH-microelectrode.

RESULTS AND DISCUSSION

In mitochondria isolated from brown adipose tissue, liver, and muscles, the uncoupling effect of low concentrations of fatty acids involves anion carriers located in the inner mitochondrial membrane (see for review [3]). It is possible that high concentrations of fatty acids increase proton conductance due to their direct (i.e., carrier-independent) transport either in protonated or anionic form through the inner mitochondrial membrane.

However, data on the effect of fatty acids on the conductance of phospholipid bilayer membrane are rather contradictive. Some authors did not find increased bilayer lipid membrane conductance using palmitate concentration, which caused uncoupling of mitochondrial respiration [11]. Others demonstrated using neutral phospholipid bilayer membranes that fatty acids caused some increase in conductance [12]. Similar results were also obtained using liposomes [13]. These discrepancies may be attributed to different experimental conditions, such as phospholipid composition of bilayer membranes, solvents used for phospholipid preparation, and fatty acids. Later it was shown that 20 μM palmitate increased conductance of bilayer lipid membrane prepared from mitochondrial phospholipids provided that phospholipids were dissolved in decane–chlorodecane mixture (but not in decane only) [14].

A similar situation was also found in proteoliposomes prepared with bovine heart cytochrome *c* oxidase.

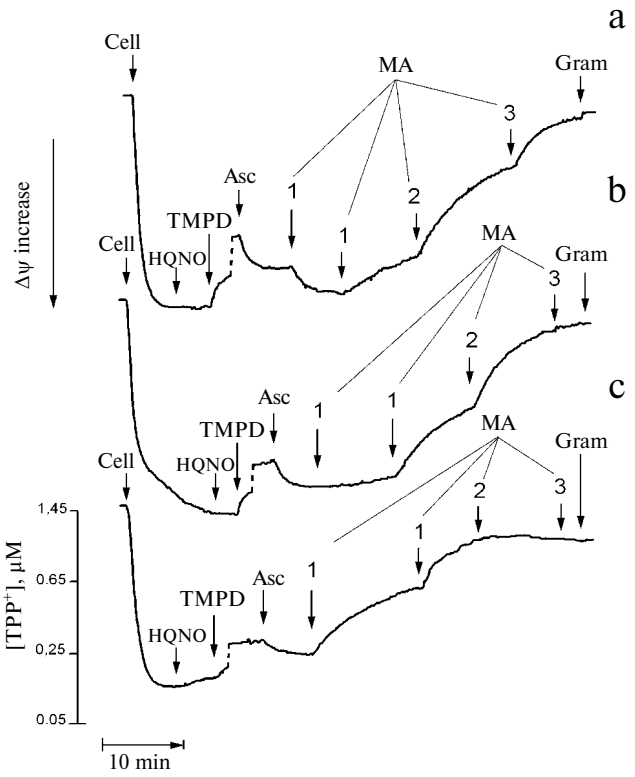

Fig. 1. Effect of myristic acid on membrane potential of *Bacillus pseudofirmus* FTU cells. The cells at logarithmic phase of growth, which were exhausted of endogenous substrates for 2 days at 4°C, were used in experiments. Changes of membrane potential were registered using the permeable cation TPP^+. a) Cells were not pretreated with DCCD. b) Before experiment cells were pretreated for 2 h at 4°C with 200 μM DCCD. c) Before experiment cells were pretreated for 2 h at 4°C with 400 μM DCCD. The assay medium contained 150 mM KCl, 450 mM NaCl, 5 mM Tris-HCl (pH 8.2). Arrows show additions to the assay medium of cell suspension (Cell); 10 mM potassium ascorbate (Asc); myristic acid (MA): 1) 10 μM; 2) 20 μM; 3) 40 μM; 1 μg/ml gramicidin D (Gram).

Some authors found that fatty acids increased membrane potential on proteoliposomes prepared from mitochondrial phospholipids [11], whereas others found that fatty acids decreased membrane potential of the same research object [15].

Figure 1a shows that low concentrations of fatty acids decreased membrane potential of the bacterial cells only after exhaustion of endogenous substrates under conditions of partial inhibition of respiratory chain by HQNO and oxidation of initial segments of respiratory chain with TMPD. (Here we show the effect of myristic acid only.) The decrease in membrane potential was registered at 30-μM concentration of fatty acid and above. The effectiveness of the uncoupling effect reduced in the following order: myristic acid > palmitic acid > lauric acid. Fatty acid-induced increase of intact cell respiration (table) can be attributed to several mechanism(s): a) uncoupling; b) dehydrogenase activation; c) increase in ATPase activity. Since membrane potential of intact cells decreases (Fig. 1, a-c), mechanism (b) can be ruled out, because net dehydrogenase activation is usually accompanied by increase in membrane potential. Also, this effect cannot be explained by ATPase activation (mechanism (c)), because it has been also observed using proteoliposomes containing cytochrome *c* oxidase in the absence of ATPase (Fig. 2, a and b). In experiments with intact cells, the fatty acid-induced decrease in membrane potential was also observed in the presence of the ATPase inhibitor DCCD (Fig. 1, b and c). This also argues against mechanism (c). Thus, the uncoupling mechanism (a) is the most probable mechanism mediating the fatty acid effect.

Results of experiments on intact cells suggest that the uncoupling effect of fatty acids can be detected only at low rate of potential generation, because in this case the respiratory chain cannot restore membrane potential up to the initial level with this type of proton shunt. Results shown in Fig. 1 (a-c) confirm this suggestion. Decrease in activity of potential generators by increasing concentrations of DCCD was accompanied by reduction of acting concentrations of myristic acid from 30 to 5-10 μM.

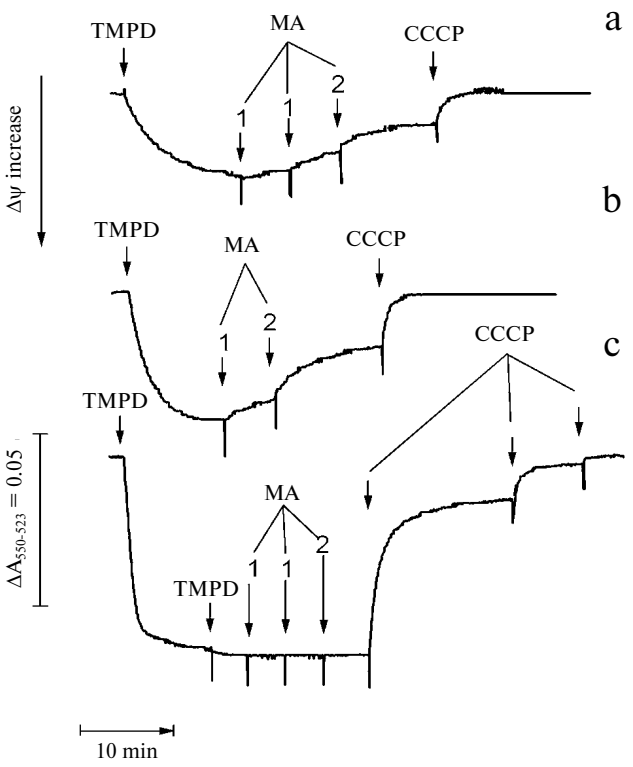

Fig. 2. Effect of myristic acid on membrane potential of proteoliposomes with reconstituted cytochrome *c* oxidase *caa*$_3$ type from *Bacillus pseudofirmus* FTU cells. Changes of membrane potential were registered using the permeable lipophilic cation safranine O. Assay medium (total volume 1 ml) contained 21 μM safranine O, 50 mM Tricine-KOH (pH 8.0), 100 mM K_2SO_4, 10 mM $MgSO_4$, 0.5 EDTA, and 5 mM potassium ascorbate. Proteoliposomes containing 1 mg of asolectin and 0.5 nmol of heme *a* of oxidase were added to the assay medium in the volume of 16 μl. Membrane potential generation was initiated by addition of 5 μM TMPD (a), 10 μM TMPD (b), and 40 μM TMPD (c) followed by subsequent addition of 20 μM TMPD. Arrows show addition of 20 μM (1) and 40 μM (2) myristic acid (MA); 300 nM CCCP (a, b) or by repeated (triple) addition of 100 nM CCCP (c).

Effect of fatty acids and the protonophore uncoupler CCCP on oxygen consumption by intact cells of *Bacillus pseudofirmus* FTU and cytochrome oxidase purified from them

Compound	Concentration	Oxygen consumption, %
Cells		
—		100
CCCP	1 μM	190
Myristic acid	5 μM	115
	20 μM	133
Palmitic acid	8 μM	120
Lauric acid	20 μM	125
Cytochrome oxidase *caa*$_3$ type		
—		100
Myristic acid	20 μM	100
	40 μM	100
	80 μM	100

Note: Respiration was measured at 22°C. Before experiment cells were preincubated with 200 μM DCCD for 2 h and then with 80 μM TMPD for 20 min. Respiration was initiated by adding 5 mM ascorbate into a polarographic cell. Oxygen consumptions of 2 and 10 μg-atoms of O/min per mg protein were defined as 100% for intact cells and cytochrome oxidase, respectively.

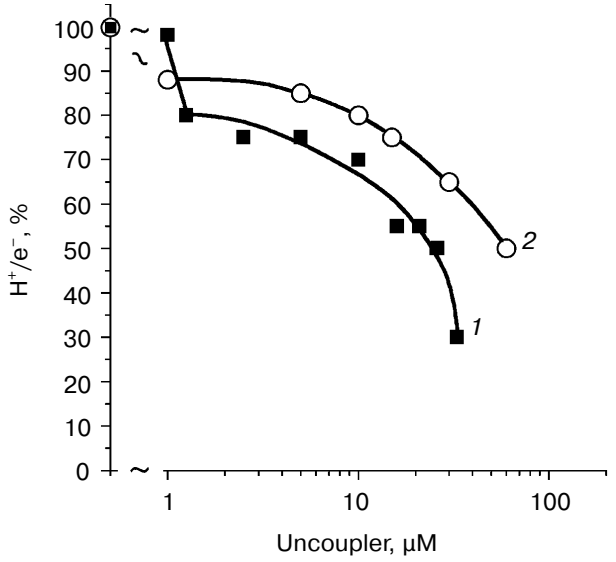

Fig. 3. Effect of myristic acid (*1*) and uncoupler DNP (*2*) on H^+ release from *Bacillus pseudofirmus* FTU cells in response to oxygen addition to anaerobic medium. Cells prepared as shown for Fig. 1 were preincubated for 20 min in medium containing 150 mM KCl, 450 mM NaCl, 1 μM valinomycin, 5 μM HQNO, 80 μM TMPD, 5 mM potassium ascorbate, and 0.5 mM Tricine-KOH, pH 8.1. Proton release from cells was induced by addition of 10 μl of water at 25°C. Changes in pH after each addition of water were calibrated by adding 10 nmol of HCl saturated with argon into the cell.

Figure 2 (a-c) shows that the rate of potential generation by cytochrome *c* oxidase in the artificial system may be evaluated by initial rate of potential increase induced by TMPD addition in the presence of ascorbate. The effect of 20-μM myristic acid on decrease in membrane potential becomes clear only when the rate of potential generation is low (Fig. 2, a and b); it is not seen at all under conditions of high rate of potential generation (Fig. 2c). Besides increased respiration and decreased membrane potential, the same fatty acid concentrations caused decrease in H^+/e^- ratio measured in intact cells (Fig. 3). Since H^+/e^- ratio was measured at the terminal site of the respiratory chain and fatty acids did not influence cytochrome oxidase activity (table), it is possible that fatty acids induced increase in H^+-conductance as was observed in liver mitochondria [16]. The alternative interpretation that in bacterial cells fatty acids cause conversion of ΔpH into $\Delta\psi$ may be excluded by the fact that fatty acids decrease rather than increase $\Delta\psi$ (Fig. 1). Thus, these experimental data clearly demonstrate that myristic acid acts as an uncoupler of the energy-coupling system in *Bacillus pseudofirmus* FTU bacterial cells.

The molecular mechanism of the fatty acid effect on bacterial cell is not understood in all details. In bacteria the involvement of such anionic carriers as ATP/ADP antiporter [11] and aspartate–glutamate antiporter [17], well known carriers of uncoupling effect of fatty acids in mitochondria, can be ruled out. The ATP/ADP antiporter has not been found in bacterial membranes. Possible involvement of the aspartate–glutamate antiporter was excluded by lack of recoupling effect of 8 mM glutamate on the uncoupling effect of micromolar concentrations of myristic acid. All our results (including the uncoupling effect of fatty acids in the reconstituted system containing purified cytochrome *c* oxidase) suggest the following mechanism of the effect of fatty acids (as protonophore uncouplers) on bacterial cells. The protonated form of the fatty acid readily crosses the lipid bilayer in both directions. In contrast to the non-ionized molecule, the anionic form of the fatty acid cannot cross the phospholipid membrane provided that the membrane hydrophobicity is not artificially reduced by chlorodecane-like compounds. At the same time, fatty acid functioning as protonophore requires transmembrane translocation of its anion. It is possible that permeability of bacterial membrane for fatty acid anions involves membrane proteins. Such permeability is rather low because the uncoupling effect of fatty acid can be observed only at low rate of electron transfer by the respiratory chain ("mild uncoupling", see for review [3]). In bacteria, biological functions of fatty acid-induced uncoupling may be the same as in higher organisms (with the exception of thermoregulatory function). It is possible that in particular bacteria "mild" oxidation by fatty acids can be specifically important for energy supply of immediate syntheses required for adaptation to constantly changing environment. Sharp rearrangement of lipid composition of membranes due to activation of fatty acid synthesis is one of the adaptive mechanisms responsible for adaptation of halo- and alkalotolerant bacteria to altered alkalinity and salinity of their environment [18]. These processes require additional supply with reducing equivalents and ATP, and they share some similarity with processes occurring in young organisms, in which the ratio between noncoupling oxidation and oxidative phosphorylation is shifted to the former [1].

This work was supported by the Russian Foundation for Basic Research (grant No. 02-04-49107) and the Ministry for Education and Science (grant "Leading Scientific Schools" No. 1710.2003.04).

REFERENCES

1. Skulachev, V. P. (1962) *Ratio between Oxidation and Phosphorylation in Respiratory Chain* [in Russian], Academic Press of the USSR, Moscow.
2. Skulachev, V. P. (1969) *Accumulation of Energy in the Cell* [in Russian], Nauka, Moscow.
3. Skulachev, V. P. (1998) *Biochim. Biophys. Acta*, **1363**, 100-124.
4. Pressman, B. C., and Lardy, H. A. (1956) *Biochim. Biophys. Acta*, **21**, 458-466.

5. Skulachev, V. P., and Maslov, S. P. (1960) *Biokhimiya*, **25**, 1058-1065.
6. Levachev, M. M., Mishukova, E. A., Sivkova, V. G., and Skulachev, V. P. (1965) *Biokhimiya*, **30**, 864-874.
7. Muntyan, M. S., Tourova, T. P., Lysenko, A. M., Kolganova, T. V., Fritze, D., and Skulachev, V. P. (2002) *Extremophiles*, **6**, 195-199.
8. Kamo, N., Muratsuga, M., Hongoh, R., and Kobatake, Y. (1979) *J. Membr. Biol.*, **49**, 105-121.
9. Huttunen, M. T., and Akerman, K. E. O. (1980) *Biochim. Biophys. Acta*, **597**, 274-284.
10. Grischuk, Yu. V., Muntyan, M. S., Popova, I. V., and Sorokin, D. Yu. (2003) *Biochemistry (Moscow)*, **68**, 385-390.
11. Andreyev, A. Yu., Bondareva, T. O., Dedukhova, V. I., Mokhova, E. N., Skulachev, V. P., Tsofina, L. M., Volkov, N. I., and Vygodina, T. V. (1989) *Eur. J. Biochem.*, **182**, 585-592.
12. Gutknecht, J. (1988) *J. Membr. Biol.*, **106**, 83-93.
13. Sharpe, M. A., Cooper, C. E., and Wrigglesworth, J. M. (1994) *J. Membr. Biol.*, **141**, 21-28.
14. Tsofina, L. M., and Mokhova, E. N. (1998) *Biosci. Rep.*, **18**, 91-95.
15. Sharpe, M., Perin, I., and Nicholls, P. (1996) *FEBS Lett.*, **391**, 134-138.
16. Brustovetsky, N. N., Dedukhova, V. I., Egorova, M. V., Mokhova, E. N., and Skulachev, V. P. (1990) *FEBS Lett.*, **272**, 187-189.
17. Samartsev, V. N., Mokhova, E. N., and Skulachev, V. P. (1997) *FEBS Lett.*, **412**, 179-182.
18. Russell, N. J. (1989) *J. Bioenerg. Biomembr.*, **21**, 93-113.