Biochemistry (Moscow), Vol. 68, No. 4, 2003, pp. 385-390. Translated from Biokhimiya, Vol. 68, No. 4, 2003, pp. 477-483.

Ion Transport Coupled to Terminal Oxidase Functioning in the Extremely Alkaliphilic Halotolerant Bacterium *Thioalkalivibrio*

Yu. V. Grischuk[1], M. S. Muntyan[1]*, I. V. Popova[1], and D. Yu. Sorokin[2]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119992, Russia; fax: (095) 939-3181; E-mail: muntyan@genebee.msu.su*
[2]*Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7/2, Moscow 117811, Russia; fax: (095) 135-6530*

Received January 16, 2002
Revision received April 4, 2002

Abstract—Proton transport in the terminal part of the respiratory chain in the extremely alkaliphilic halotolerant bacterial strain *Thioalkalivibrio versutus* was studied under near-optimum growth conditions (pH 9.0-9.5). Under these conditions, bacterial cells generated electric potential with the negative charge being inside the cells. When only the terminal part of the respiratory chain functioned, it was found that: 1) unlike other bacteria known, this bacterium did not acidify the medium in the presence of K^+ and valinomycin; 2) in the presence of an uncoupler, CCCP, but in the absence of valinomycin, reversible alkalinization of the medium occurred as a result of proton influx into the cells. Cyanide prevented this alkalinization. The difference spectra indicate that cell membranes contained cytochromes *c* and (*b*+*o*), some of which reacted with CO. The respiratory activity of membranes in the terminal part of the respiratory chain was optimal at pH 9.5 and specifically depended on sodium ions ($C_{1/2}$ = 10 mM). The data suggest the presence of a Na^+-pump in the terminal part of the respiratory chain of the studied strain which can pump Na^+ out of the cells.

Key words: terminal oxidase, Na^+ transport, extremely alkaliphilic bacteria, halotolerance, *Thioalkalivibrio*

Currently, extremely alkaliphilic microorganisms attract the attention of bioenergetics researchers because they contain unique energy-coupling systems. One of these microorganisms is a novel chemolithotrophic halotolerant (resisting 2 M Na^+) sulfur-oxidizing bacterium *Thioalkalivibrio versutus* with pH optimum at 9.5-10, which was discovered in alkaline soda lakes in southeastern Siberia (Republic of Tuva) [1]. Phylogenetic studies showed that the representatives of obligate aerobic bacteria belonging to the genus *Thioalkalivibrio* are only remotely related to anaerobic purple sulfur-oxidizing bacteria of the genus *Ectothiorhodospira* and apparently represent a separate evolutionary branch [2]. Such microorganisms (whose mechanisms of energy storage are poorly studied thus far) may serve as objects for searching for new enzyme complexes of the respiratory chain with sodium rather than proton (Mitchell's) energy-coupling type. The ability to store energy in the form of a Na^+ gradient on the membrane is characteristic of some anaerobic and marine alkaliphilic bacteria [3]. In these bacteria, the enzymes of the first energy-coupling site of the respiratory chain, F_oF_1- or V_oV_1-ATPases and decarboxylases, can use Na^+ as a coupling ion [4-6]. During the past decade researchers have sought for a Na^+-transporting enzyme in complex IV of the respiratory chain; however, no significant results have been obtained. This work is devoted to this problem.

MATERIALS AND METHODS

Bacterial culture. *Thioalkalivibrio versutus* AL2 was cultured under aerobic conditions in a circulatory shaker (200 rpm) at 37°C in liquid nutritional medium (250 ml per flask) containing 23 g/liter Na_2CO_3, 7 g/liter $NaHCO_3$, 6 g/liter NaCl, 0.5 g/liter KNO_3, 1 g/liter KH_2PO_4, 60 mM $Na_2S_2O_3$, 2 ml/liter microelements [7],

Abbreviations: DTT) dithiothreitol; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; FCCP) carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HQNO) 2-heptyl-4-hydroxyquinoline N-oxide; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

* To whom correspondence should be addressed.

0006-2979/03/6804-0385$25.00 ©2003 MAIK "Nauka/Interperiodica"

and 1 mM $MgCl_2 \cdot 6\ H_2O$ (pH 10.0). All components of the medium, except for thiosulfate, microelements, and $MgCl_2$, were autoclaved together. The growth of culture was estimated by the extent of thiosulfate consumption by the cells, which was monitored by titration of a culture aliquot with solution containing 0.01 N I_2 and 5% acetic acid. The culture was considered grown at a titer less than 0.2 ml I_2 per 0.5 ml sample, which corresponds to a residual concentration of thiosulfate in the culture less than 6 mM.

Preparation of cell suspension for experiments. Before experiments, grown culture was washed free from culture medium by centrifugation (9000*g*, 10 min) three times with buffer A containing 220 mM Na_2CO_3, 80 mM $NaHCO_3$, 100 mM NaCl, and 50 mM KCl (pH 10.3). Cell biomass was resuspended in the same buffer to a final volume equal to 0.01 volume of the initial culture. Before measuring membrane potential and H^+ release from intact cells, washed cell suspension was exhausted with respect to endogenous substrates at 4°C for 2 days and then incubated in measurement medium for 3 h.

Isolation of membrane particles of *Tv. versutus* AL2. When a cell culture reached the growth stage required, the cells were pelleted by centrifugation (10,000*g*, 10 min, 4°C) and washed twice with buffer A. Wet biomass obtained was frozen and stored at −70°C or used immediately for obtaining membrane particles. For this purpose, the cells were resuspended in medium B containing 0.05 M MOPS-KOH (pH 7.6) and 0.5 mM EDTA. Before cell destruction, DNase I (20 μg/ml) and phenylmethylsulfonyl fluoride (final concentration 1 mM) were added to the suspension. Thereafter, cell suspension was passed twice through a French press cooled to 4°C (at a pressure of 1080 atm). Unbroken cells and cell debris were removed by centrifugation (10,000*g*, 10 min, 4°C). The membrane particles contained in the supernatant were pelleted by centrifugation (200,000*g*, 2 h, 4°C), washed with medium B, and resuspended in medium B supplemented with 25% glycerol to a final protein concentration of 60 mg/ml. The particles obtained were stored at −70°C.

Preparation of proteoliposomes. Azolectin was homogenized in buffer containing 200 mM MOPS-NaOH (pH 7.4) and 55 mM *n*-octyl-β-D-glucopyranoside (20 mg azolectin per ml buffer). The suspension obtained was sonicated using an UZDN-2T ultrasonic disintegrator (22 kHz, 5-6 times, ten 1-sec periods). Cytochrome *c* oxidase of caa_3-type from bacteria *Bacillus pseudofirmus* FTU (0.36 mg/ml) was added to sonicated azolectin solution, and the mixture was stirred on a magnetic stirrer at 20°C for 15 min. The detergent was removed by stepwise addition of the sorbent Bio-Beads (Bio-Rad, USA). The liposome suspension obtained was diluted 10-fold with 200 mM KCl and centrifuged (200,000*g*, 2 h, 4°C). Pelleted proteoliposomes were resuspended in a Potter homogenizer in a minimal volume of 200 mM KCl. Cytochrome *c* oxidase from the *B. pseudofirmus* FTU membranes was obtained as described in [8].

Membrane potential (Δψ) recording. To measure Δψ in intact cells, we used penetrating cation safranine O at the concentration of 8 μM. The decrease in the safranine O concentration in measurement medium and its accumulation in the cells was monitored by the difference in absorption at 555 and 523 nm on an Aminco DW-2000 spectrophotometer in double-beam mode. The solution was aerated by means of a magnetic stirrer. Absorption of the medium (1 ml) was measured in a quartz cuvette (10 × 10 mm).

Respiratory activity of membranes was measured at 25°C on an LP7e polarograph equipped with a standard platinum Clark electrode.

Spectral analysis of cytochromes in membranes was performed on a Hitachi U-3400 spectrophotometer at room temperature.

Measurement of H^+ release from intact cells and proteoliposomes. The ion-transport activity of oxidase complexes of the respiratory chain in anaerobic suspension of *Tv. versutus* AL2 cells or proteoliposomes in response to oxygen addition was assessed by the change in pH of the medium using a combined glass pH microelectrode and a pH meter (Ionomer I-130, Gomel', Belarus) connected with a PC. Before the experiment, cell suspension or proteoliposomes (1 ml) suspended in 0.62 M or 0.15 M KCl, respectively, was placed in 1-ml anaerobic measurement well on a magnetic stirrer. Thereafter, 1 μM valinomycin, 5 μM HQNO, 5 mM ascorbate-KOH (pH 7.0), and 100 μM TMPD were added to cell suspension. In the case of proteoliposomes, the solution contained 1 μM valinomycin, 5 mM ascorbate-KOH (pH 7.0), 10 μM horse heart cytochrome *c*, and 10 μM TMPD. Respiration of cell suspension or proteoliposomes preincubated under anaerobic conditions was initiated by addition of water (5-10 μl) saturated with air oxygen at 25°C. The changes in pH in the measurement well occurred in response to oxygen addition were determined by titration with 1 mM HCl saturated with argon.

Protein content was determined by the method of Lowry in the modification of Markwell [9] using BSA as a standard.

RESULTS AND DISCUSSION

The type of energy coupling in *Tv. versutus* AL2 in the terminal part of the respiratory chain was determined in the presence of HQNO, an inhibitor of complexes I and III of the respiratory chain, by evaluation of the H^+/e^- ratio after addition of small volumes of oxygen in anaerobic suspension of living cells. A prerequisite for use of this approach is exclusion of the buffer components from the measurement medium. In the measurement

medium selected, which meets this demand, the cells generate electric membrane potential ($\Delta\psi$), with the negative charge being located inside the cells (similar to neutrophilic and alkali-resistant bacteria) and $\Delta\psi$ abolished by low concentrations of an uncoupler, protonophore FCCP (Fig. 1). Under the same conditions, in the presence of ascorbate and TMPD (serving as electron donors) and K^+ ionophore, valinomycin, the cells in anaerobic suspension either do not acidify the medium or slightly alkalize it in response to O_2 addition (which reflects H^+ influx to the cell with the H^+/e^- stoichiometry of 0.15) (Fig. 2a). In the presence of an uncoupler, the protonophore CCCP (8 μM), an addition of O_2 results in alkalinization of the medium ($H^+/e^- = 0.4$) without pH relaxation (Fig. 2b). With regard for the fact that 0.5 H^+/e^- is released to the medium as a result of oxidation of ascorbate to monodehydroascorbate, these data indicate that approximately 1 H^+/e^- is consumed by the cells in the course of respiration (see below). In the same system and under the same conditions, in the presence of ascorbate, TMPD, and valinomycin, the alkali-resistant bacterium *B. pseudofirmus* FTU, whose membranes contain an H^+-pump (*caa*$_3$-type oxidase), acidifies the incubation medium (with the H^+/e^- ratio of 1.3) in response to oxygen addition. A simplified artificial system comprised of proteoliposomes with integral H^+-pump (*caa*$_3$-type cytochrome *c* oxidase from *B. pseudofirmus* FTU) served as an additional control. In this system, when cytochrome *c*, ascorbate, and TMPD were used as substrates, the H^+/e^- ratio in the presence of valinomycin was 1.5 at pH 7.0 and changed insignificantly upon increasing pH from 7.0 to 9.8. Taking into consideration that 0.5 H^+/e^- is released into the medium during ascorbate oxidation, it can be concluded that cytochrome oxidase from *B. pseudofirmus* FTU carries approximately 1 H^+/e^- across the membrane to the medium. Thus, H^+ release by cytochrome oxidase in proteoliposomes upon oxygen reduction does not depend on pH within the pH range from 7.0 to 9.8. The study of intact *Tv. versutus* AL2 cells showed that, under the measurement conditions used, the main enzyme contained in the terminal part of the respiratory chain, apparently, does not pump H^+ from the cells.

In independent experiments, we used DTT instead of potassium ascorbate as a substrate capable of TMPD reduction. With regard for the low redox potential of DTT (–0.33 V, pH 7.0), it cannot be ruled out that the substrates of complexes I and III of the respiratory chain may be reduced and, hence, involved in the reactions related to O_2 reduction. The control experiments showed that in the presence of the sole reducer, DTT, an aerobic cell suspension does not consume oxygen. Activation of respiration requires the presence of TMPD, which allowed us to exclude the contribution of complexes I and III to the ion transport recorded. With regard for this fact, it can be assumed that, in the presence of DTT and TMPD, only

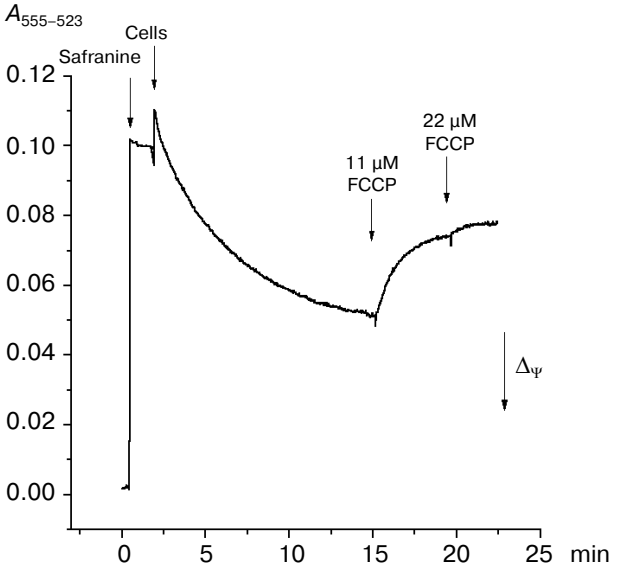

Fig. 1. Spectrophotometric measurement of $\Delta\psi$ in *Tv. versutus* AL2 cells using safranine O. Incubation medium contained 0.62 M KCl and 5 μM HQNO (pH 9.0).

the terminal part of the respiratory chain functions in the cells studied. Under these conditions, anaerobic suspension of *Tv. versutus* AL2 cells alkalinized the medium ($H^+/e^- = 1.0$ at pH 8.3) in the presence of CCCP in response to oxygen addition. In this case, alkalinization is accompanied by a rapid pH relaxation to the initial value (Fig. 3a). A rapid alkalinization of the medium with a stoichiometry $H^+/e^- = 1.3$ is also observed at pH 9.0 (Fig.

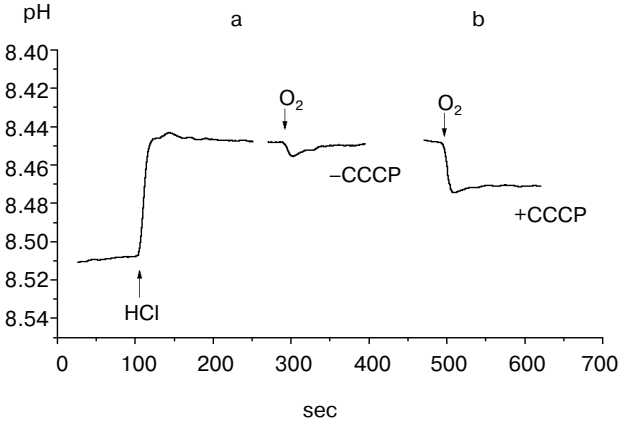

Fig. 2. Estimation of the efficiency of the proton pump in the terminal part of the respiratory chain in *Tv. versutus* AL2 cells by oxygen additions to anaerobic cell suspension. Measurement medium contained 0.6 M KCl, 5 mM ascorbate-KOH (pH 7.0), 100 μM TMPD, 1 μM valinomycin, 20 mM NaCl, 5 μM HQNO (a) and 8 μM CCCP (b). Oxygen was added to anaerobic cell with water (10 μl). The oxygen-induced changes in pH of the cell suspension were calibrated by addition of HCl (10 nmol).

3b). However, the medium becomes more alkaline compared to the initial value due to pH relaxation. As a result of alkalinization of the medium by a value corresponding to the difference between the initial and final pH values, the H^+/e^- stoichiometry is 0.35. Alkalinization is completely abolished by addition of 2 mM cyanide to the cell suspension (Fig. 3c).

Any terminal oxidase, irrespective of whether or not it is an ion pump, consumes 4 H^+ from the inner space of the cell during O_2 reduction to two H_2O molecules. In the case of cytochrome *c* oxidase, with cytochrome *c* (which does not change H^+ balance in the medium during oxidation and reduction) serving as an electron donor, catalysis is accompanied by intracellular alkalinization with a stoichiometry $H^+/e^- = 1$. Intracellular alkalinization is irreversible; therefore, it should not yield pH relaxation during the measurement (as in the case of acidification during DTT oxidation). Assuming that oxidation of either of the two SH groups of DTT does not affect p*K* of the other SH group (DTT dissociates as a dibasic acid with two p*K* values equal to 8.3 and 9.5), acidification of medium as a result of DTT oxidation at pH 8.3 and 9.0 will have a stoichiometry $H^+/e^- = 0.75$ and ~0.5 and should be irreversible. It can be assumed that irreversible intracellular alkalinization at pH 8.3 is predominantly compensated due to acidification produced as a result of DTT oxidation. Therefore, the contribution of irreversible alkalinization is either absent (Fig. 3a) or accounts for only a small portion of the entire response and hence is not recorded. In view of this, reversible alkalinization of the medium shown in Fig. 3a may be considered as a reversible influx into the cell of approximately 1 H^+ per electron transferred to oxygen.

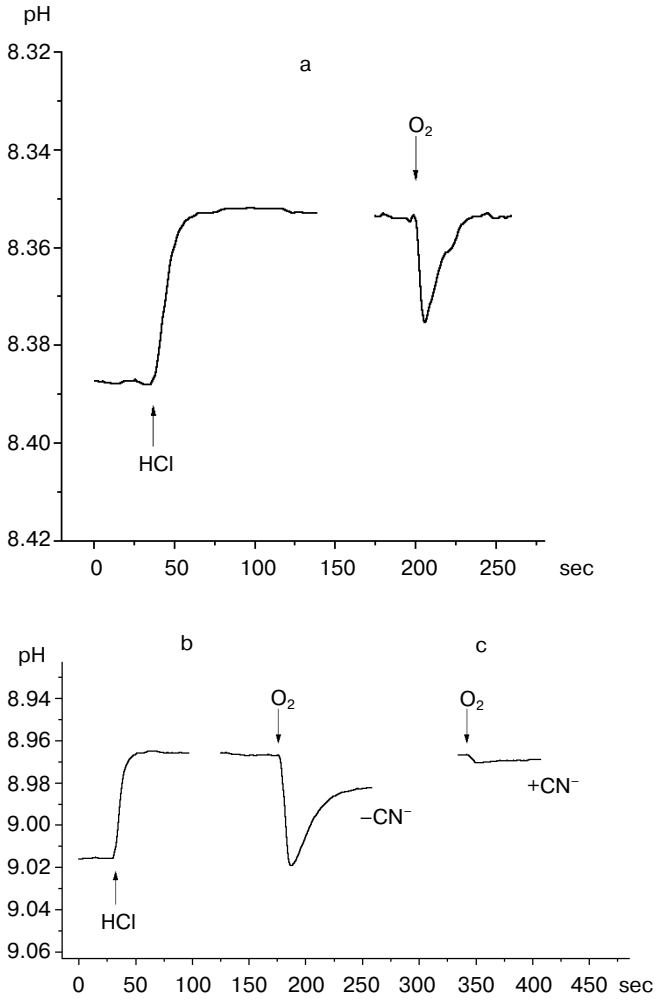

Fig. 3. Estimation of the efficiency of the proton pump functioning in the terminal part of the respiratory chain in *Tv. versutus* AL2 cells by oxygen additions to anaerobic cell suspension at pH 8.3 (a) and 9.0 (b). Measurement medium contained 0.12 M KCl, 0.2 mM DTT, 100 μM TMPD, 0.5 M NaCl, 5 μM HQNO, 80 μM CCCP (a, b), and 2 mM KCN (c). Oxygen was added to the anaerobic cell with water (10 μl). The oxygen-induced changes in pH of the cell suspension were calibrated by addition of HCl (10 nmol).

As seen from Fig. 3b, at pH 9.0, when acidification of the medium resulting from DTT oxidation decreases compared to acidification observed at pH 8.3, an irreversible fraction occurs in the overall alkalinization of the medium produced by the cell suspension. If presumably irreversible intracellular alkalinization caused by oxidase is equivalent to the consumption of 1 H^+ per electron transferred, and acidification of the medium resulting from DTT oxidation at pH 9.0 is approximately 0.5 H^+/e^-, then actual irreversible alkalinization due to O_2 reduction should be equal to approximately 0.5 H^+/e^-. In fact, it is approximately 0.35 H^+/e^- (Fig. 3b), which is a good approximation to the calculated value. The reversible portion of alkalinization at pH 9.0 (Fig. 3b) is equivalent to influx of approximately 0.9 H^+/e^- into the cell from the medium.

Thus, the calculations and the data shown in Fig. 3 (a and b) indicate that at least 0.9 H^+/e^- reversibly enters the cell upon the transfer of one electron from the substrate to O_2. With regard for the principle of electric neutrality, this is indicative of pumping out of the cell of positively charged ions, other than proton (for example, sodium or potassium ions, which are contained at high concentrations in culture medium). In this case, specific activation of respiration by the ions pumped from the cell could be expected. This assumption was checked using membrane preparations.

Tv. versutus AL2 membranes are characterized by a high respiratory activity (up to 2 μmol O_2/min per mg protein) with pH optimum at 9.5 (Fig. 4). We found that the respiratory activity completely depended on the presence of monovalent cations in the medium. The mode of dependence on Na^+ and K^+ is different (Fig. 5). The curve of dependence on Na^+ is biphasic. The activity in phase 1 sharply increased up to 0.8 μmol O_2/min per mg

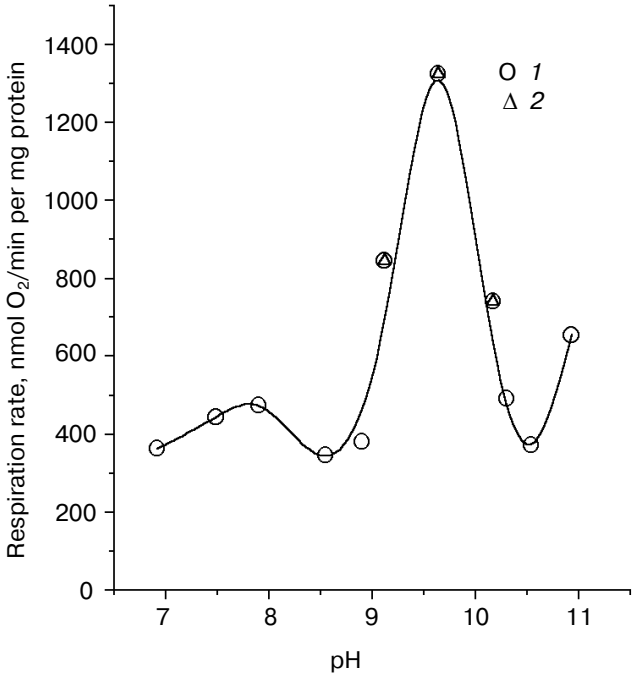

Fig. 4. Dependence of the respiratory activity of *Tv. versutus* AL2 membranes on pH in the absence (*1*) and presence (*2*) of 5 μM HQNO. Measurement medium contained 40 mM $NaHCO_3$, 40 mM Tris, 40 mM Tricine, 40 mM MOPS, 100 mM NaCl, 100 μM TMPD, and 5 mM ascorbate (pH was adjusted with KOH).

protein as $[Na^+]$ in medium increased to 20 mM. Thereafter, it increased relatively slowly (a twofold increase, compared to the previous level, was reached when $[Na^+]$ increased more than tenfold, i.e., to 250 mM). Note that K^+, added at the same concentrations that cause a pronounced increase in the respiratory activity in the case of Na^+, has no effect and left respiration in the latent state. Beginning from a concentration of 20 mM, K^+ activated respiration in a manner similar to the second phase of the curve of dependence on Na^+ concentration. Thus, it can be assumed that low concentrations of Na^+ (less than 20 mM) activate respiration in the terminal part of the respiratory chain in a specific manner; beginning from a concentration 20 mM, monovalent cations nonspecifically activate respiration due to a salt effect. It is known that terminal quinol oxidases in bacteria may be inactivated in the presence of HQNO [10, 11]. In view of this, it cannot be ruled out that, in experiments performed with intact cells in the presence of HQNO, Na^+-activated terminal oxidase was blocked (Figs. 2 and 3). Note that TMPD used in the experiments may serve as an electron donor both for quinol oxidases and cytochrome *c* oxidases. Therefore, a probability exists that the respiratory activity of membranes measured in the terminal part of the respiration chain is ensured by both oxidase types. The absence of sensitivity of Na^+-activated terminal oxidase in membranes of the bacterium studied to HQNO (Fig. 4) assumes that Na^+-activated terminal oxidase is involved in alkalinization of the incubation medium by intact bacterial cells in response to oxygen addition in the presence of HQNO.

It was interesting to compare the data on the respiration activation by monovalent cations with similar characteristics of Na^+-transporting enzymes of other types. Na^+-ATPase from the anaerobic bacterium *Propionigenium modestum* is activated by 5 mM Na^+ (K_m = 0.7 mM) [12]. Na^+-ATPase from another anaerobic bacterium, *Acetobacterium woodii*, is activated by 2 mM NaCl [13]. Na^+-transporting NADH:quinone oxidoreductase from membranes of marine halotolerant bacterium *Vibrio alginolyticus* is activated by Na^+ with K_m = 80 mM [14]. In the case of Na^+-oxaloacetate decarboxylase from the pathogenic bacterium *Klebsiella pneumoniae*, K_m (Na^+) = 0.5 mM [15]. In *Vitreoscilla* sp. C1 membranes, ubiquinone oxidase activity is stimulated by 80 mM NaCl [16]. In all cases, KCl did not have such a stimulatory effect on the activity of the enzymes as NaCl.

Spectral analysis of membrane preparations of *Tv. versutus* AL2 showed that *a*- and *d*-type cytochromes cannot serve as Na^+-activated terminal oxidases in this

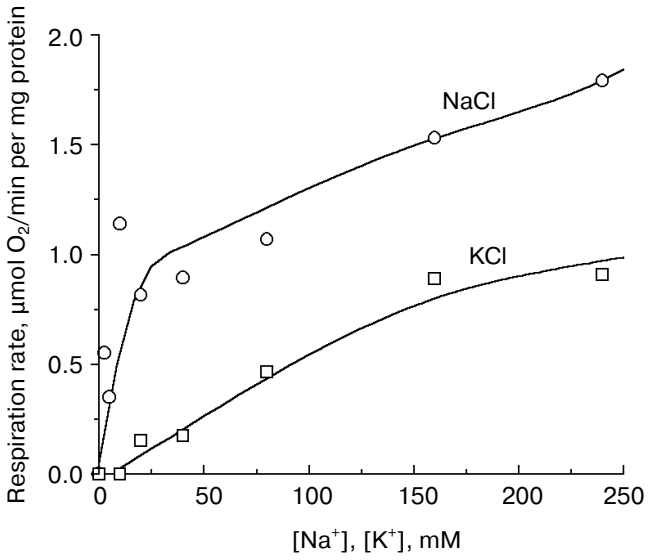

Fig. 5. Dependence of the respiratory activity of *Tv. versutus* AL2 membranes on the concentration of monovalent cations in the medium. Measurement medium contained 50 mM Tris-HCl (pH 9.5) and 0.5 mM EDTA. Monovalent cations were added to the polarographic cell (prior to the addition of the respiration substrates and membranes) in the form of NaCl or KCl solutions with the initial concentration of 1-2 M. Ascorbic acid and TMPD at final concentrations 5 mM and 100 μM, respectively, were used as substrates for respiration. Respiration was induced by addition of membranes (to a final concentration of 34 μg protein/ml) to the polarographic cell.

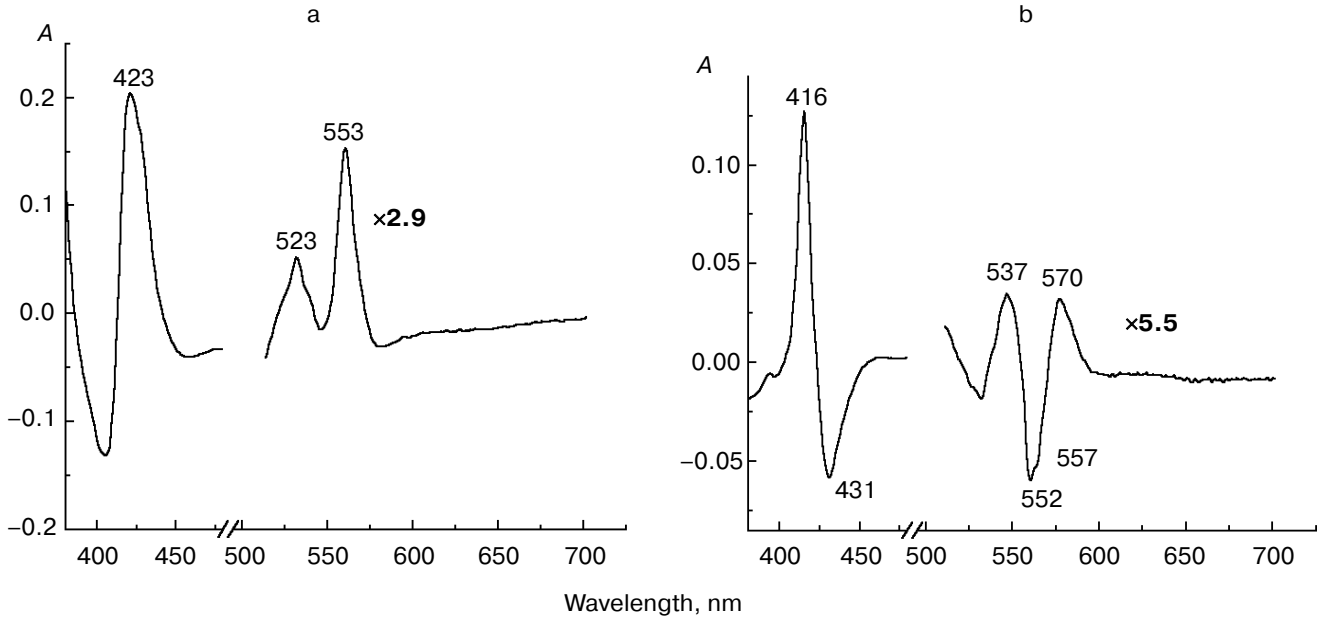

Fig. 6. Difference absorption spectra of *Tv. versutus* AL2 cells: reduction with sodium dithionite minus oxidation with air (a) and CO-reduction with sodium dithionite minus reduction with sodium dithionite (b).

strain. The difference spectra of membranes (reduced-minus-oxidized) shown in Fig. 6a allowed us to reveal cytochromes *c* and (*b*+*o*). As clearly seen from the CO difference spectra (Fig. 6b), *c*- and *bb*-type cytochromes bind CO (the characteristics of CO-binding cytochromes of *bb*-type are described in the article of Muntyan et al. [17]). Possibly, they contain a fraction of cytochrome *o*. These data suggest that *bb*- and/or *o*-type cytochromes could serve as Na^+-dependent terminal oxidases in *Tv. versutus* AL2.

This work was supported by the Russian Foundation for Basic Research (project No. 02-04-49107).

REFERENCES

1. Sorokin, D. Yu., Robertson, L. A., and Kuenen, J. G. (2000) *Antonie van Leeuwenhoek*, **77**, 251-262.
2. Sorokin, D. Y., Lysenko, A. M., Mityushina, L. L., Tourova, T. P., Jones, B. E., Rainey, F. A., Robertson, L. A., and Kuenen, G. J. (2001) *Int. J. Syst. Evol. Microbiol.*, **51**, 565-580.
3. Skulachev, V. P. (1994) *J. Bioenerg. Biomembr.*, **26**, 589-598.
4. Skulachev, V. P. (1999) *Novartis Found. Symp.*, **221**, 200-217.
5. Lolkema, J. S., Speelmans, G., and Konings, W. N. (1994) *Biochim. Biophys. Acta*, **1187**, 211-215.
6. Häse, C. C., Fedorova, N. D., Galperin, M. Y., and Dibrov, P. A. (2001) *Microbiol. Mol. Biol. Rev.*, **65**, 353-370.
7. Pfennig, N., and Lippert, K. D. (1966) *Arch. Microbiol.*, **55**, 245-256.
8. Muntyan, M. S., Ustiyan, V. S., Viryasov, M. B., and Skulachev, V. P. (1995) *Biochem. Biophys. Res. Commun.*, **207**, 55-61.
9. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Analyt. Biochem.*, **87**, 207-210.
10. Lemma, E., Simon, J., Schagger, H., and Kroger, A. (1995) *Arch. Microbiol.*, **163**, 432-438.
11. Gartner, P. (1991) *Eur. J. Biochem.*, **200**, 215-222.
12. Laubinger, W., and Dimroth, P. (1988) *Biochemistry*, **27**, 7531-7537.
13. Reidlinger, J., and Müller, V. (1994) *Eur. J. Biochem.*, **223**, 275-283.
14. Unemoto, T., and Hayashi, M. (1989) *J. Bioenerg. Biomembr.*, **21**, 649-662.
15. Jockel, P., Schmid, M., Steuber, J., and Dimroth, P. (2000) *Biochemistry*, **39**, 2307-2315.
16. Kim, K. J., Chi, P. Y., Hwang, K. W., Stark, B. C., and Webster, D. A. (2000) *J. Biochem.* (Tokyo), **128**, 49-55.
17. Muntyan, M. S., Dinarieva, T. Yu., Baev, M. V., and Netrusov, A. I. (2002) *Arch. Biochem. Biophys.*, **398**, 118-124.