Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 113-118

ISOLATION OF VIBRIO ALGINOLYTICUS MUTANTS DEFECTIVE IN THE RESPIRATION-COUPLED Na^+ PUMP

Hajime Tokuda

Research Institute for Chemobiodynamics, Chiba University, 1-8-1 Inohana, Chiba, Japan

Received May 23, 1983

When the respiration-coupled Na^+ pump functions, V. alginolyticus is able to grow in the presence of an extremely high concentration of proton conductor, carbonylcyanide m-chlorophenylhydrazone. The mutants which became sensitive to the proton conductor were isolated and examined in regard to the Na^+ pump activity. Although the activity of a respiration-dependent H^+ extrusion by the mutants is comparable to that by the wild type, the Na^+ pump activity of the mutants is significantly reduced. Furthermore, NADH oxidase of membranes isolated from the mutants is altered to be independent of Na^+. It is concluded that the mutants have an alteration in the respiratory chain which simultaneously results in a lack of the Na^+ pump.

Our recent studies revealed that the marine bacterium V. alginolyticus extrudes not only H^+ but also Na^+ as an immediate result of respiration (1, 2). Although the extrusion of H^+ by the respiratory chain occurred at all physiological pH values tested, the respiration-coupled Na^+ extrusion took place mainly at alkaline external pH. Since the Na^+ pump generates an electrochemical potential of Na^+ even in the presence of CCCP, not only active transport of solutes(1, 2) but also growth[1] itself became resistant to CCCP at alkaline pH.

The molecular mechanism of the respiration-coupled Na^+ pump is of great interest since it may give another insight into the mechanism of the respiration-coupled H^+ extrusion which is presently a subject of controversy

[1] Tokuda, H., and Unemoto, T., manuscript in preparation.

Abbreviations: CCCP, carbonylcyanide m-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium ion; TMPD, N,N,N',N'-tetramethyl p-phenylene diamine; MES, 2-(N-morpholino)ethanesulfonic acid; Tricine, tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; NTG, N-methyl-N'-nitro-N-nitrosoguanidine.

0006-291X/83 $1.50

Copyright © 1983 by Academic Press, Inc.
All rights of reproduction in any form reserved.

(3, 4). From this reason, the isolation of mutants defective in the Na^+ pump was attempted.

MATERIALS AND METHODS

V. alginolyticus 138-2 and its derivatives were grown aerobically at 37°C on either a rich (5) or a synthetic medium(6) buffered as specified. Mutants unable to grow at pH 8.5 on the rich medium containing 5 μM CCCP were induced by NTG (50 μg/ml) at 37°C for 10 min. The mutants were isolated after two cycles of penicillin ($8x10^3$ units/ml) enrichment (7) in the rich medium containing 5 μM CCCP and 50 mM Tricine-NaOH, pH 8.5. Selection was performed by replica plating. Glucose was omitted from the seletive plates to minimize a pH drop during growth.

H^+ flux induced by oxygen pulse and active extrusion of Na^+ were assayed as reported(2) using K^+-depleted and Na^+-loaded cells prepared as described (8) from cells grown on the synthetic medium.

Membrane fractions were isolated as described(5) from cells grown on the synthetic medium. NADH oxidase activity was determined by following the decrease in absorbance at 340 nm as described(5).

$^{22}NaCl$ (carrier-free) was obtained from NEN. CCCP and NADH (sodium salt) were purchased from Sigma. MES and Tricine were products of Nakarai Chemical Co.

RESULTS

The rationale for the isolation of mutants defective in the respiration-coupled Na^+ pump was based on our finding[1] that the growth of V. alginolyticus becomes highly resistant to CCCP when the Na^+ pump functions. Close correlation between CCCP-resistant growth and the Na^+ pump was confirmed by the subsequent finding that V. costicola, another halophilic bacterium, also possesses an analogous Na^+ pump to that of V. alginolyticus and is able to grow in the presence of CCCP. Therefore, mutants lacking the Na^+ pump were expected to show CCCP-sensitive growth.

Mutants unable to grow on CCCP-containing medium were obtained by NTG mutagenesis and enrichment by penicillin in the presence of 5 μM CCCP at pH 8.5. As shown in Table 1, the minimal inhibitory concentrations for CCCP at pH 6.5, where the Na^+ pump has little activity, were not significantly different between the wild type and the isolated mutants, designated Nap 1 and Nap 2. On the other hand, at pH 8.5, where the Na^+ pump has a maximum activity, the minimal inhibitory concentrations determined with the mutants were significantly lower than that determined with the wild type. Although results are not presented, concentrations of CCCP necessary to make membranes permeable to H^+ were similar among the strains tested. The isolated mutants

Table 1. Minimal inhibitory concentration for CCCP. Aerobic growth of the listed strains in the presence of various concentrations of CCCP was examined in the rich medium buffered with 50 mM of either MES-NaOH, pH 6.5, or Tricine-NaOH, pH 8.5.

Strain	CCCP (μM)	
	pH 6.5	pH 8.5
Wild	4	>80
Nap1	3	14
Nap2	2	11

were able to grow on sucrose but not on lactose as the sole source of carbon and required Na^+ for growth. These properties were identical to those of the wild type. The spontaneous revertants were obtained at the frequency of 10^{-8}.

Extrusion of H^+ and Na^+ by the respiratory chain of three strains were examined at pH 8.5 by oxygen pulse method as reported (2). When oxygen was pulsed to anaerobic cell suspensions containing membrane-permeable TPP^+ which was added to facilitate the H^+ extrusion against its electrical potential, a rapid and transient extrusion of H^+ occurred to similar extent in all the strains tested (Fig. 1, A to C). These results indicate that the ability of mutants to extrude H^+ was comparable to that of the wild type. When oxygen was pulsed in the presence of CCCP instead of TPP^+, anaerobic suspensions of

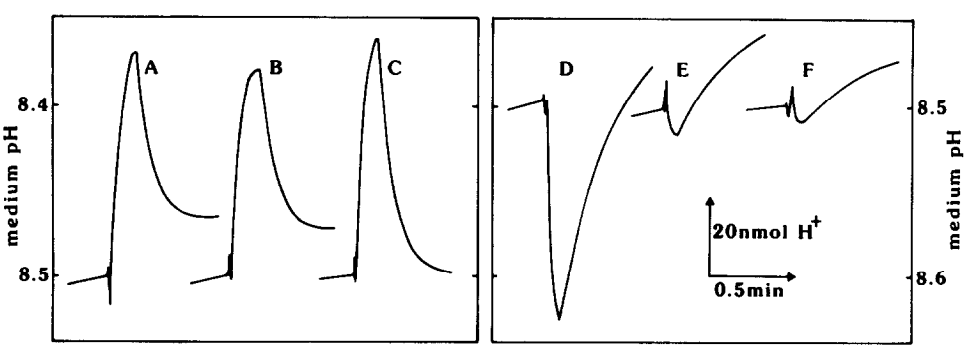

Fig. 1 Respiration-dependent H^+ extrusion (A to C) and Na^+ pump-dependent H^+ uptake (D to F). Na^+-loaded cells of the wild type (A and D), Nap 1 (B and E) and Nap 2 (C and F) were resuspended at final concentrations of 0.52, 0.66 and 0.59 mg protein/ml, respectively, in 2 ml of assay mixture containing 0.4 M NaCl, 20 mM glycerol, 0.2 mM Tricine-NaOH, pH 8.5, and either 2.5 mM TPP^+ (A to C) or 20 μM CCCP (D to F). The cell suspensions were kept anaerobic at 25°C under the stream of nitrogen. Changes in medium pH were monitored by pH-electrode as reported (2). Oxygen pulse was performed at pH 8.5 by the addition of 100 μl of air-saturated 0.4 M NaCl.

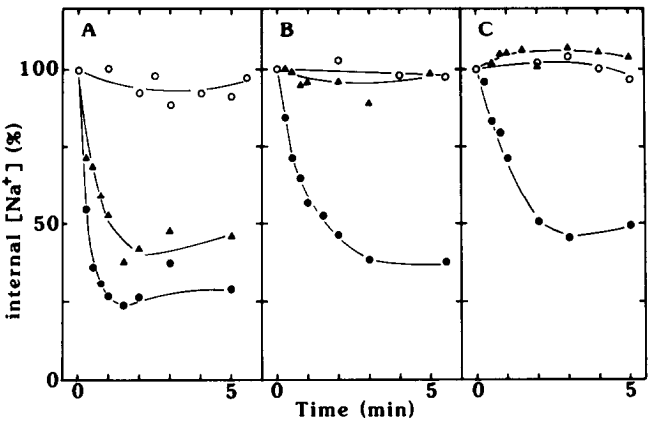

Fig. 2 Sensitivity of Na^+ extrusion to CCCP. Na^+-loaded cells of the wild type (A), Nap 1 (B) and Nap 2 (C) were resuspended at final concentrations of 3.09, 2.31 and 2.40 mg protein/ml, respectively, in 50 mM Tricine-NaOH, pH 8.5, containing 0.4 M NaCl. The cell suspensions equilibrated with $^{22}NaCl$ ($1x10^4$ cpm/µl) on ice were transferred to 25°C and incubated for 5 min in the presence of 20 mM glycerol. At 0 time, addition of none (○), 10 mM KCl (●) or 10 mM KCl plus 10 µM CCCP (▲) was made. The level of $^{22}Na^+$ retained by cells was determined at given times by filtering and washing an aliquot (50 µl) of the cell suspension as reported (1, 2). Values are given in per cent of radioactivity at 0 time after correction for background radioactivity due to non-specific binding to filters.

the wild type (Fig. 1, D) demonstrated the characteristic H^+ uptake which was driven by the Na^+ pump-generated membrane potential (2). In contrast, such a Na^+ pump-dependent H^+ uptake decreased significantly in the case of mutants (Fig. 1, E and F).

As reported previousy (1, 2) and shown in Fig. 2, K^+-depleted and Na^+-loaded *V. alginolyticus* quickly extruded most of cytoplasmic Na^+ against its concentration gradient upon the addition of K^+ (closed circles). In the wild type, a considerable portion of this Na^+ extrusion was performed by the Na^+ pump at pH 8.5 and, therefore, insensitive to CCCP (triangles in A). In contrast, the extrusion of Na^+ by the mutants (B and C) was completely inhibited by CCCP. These results clearly indicate that the isolated mutants are defective in the Na^+ pump and that the extrusion of Na^+ by the mutants is performed only by a CCCP-sensitive Na^+/H^+ antiport system.

Possible alteration in the respiratory chain of the mutants was examined in membrane fractions (Table 2). The NADH oxidase of the wild type specifically required Na^+ for maximum activity as reported (5). K^+, or other

Table 2. NADH oxidase activity of membranes isolated from the wild type and the mutants. NADH oxidase activity was measured at 30°C as described under "Materials and Methods" in 1 ml of assay mixture containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.2 M of KCl or NaCl. The assay was started by the addition of membranes to give a final concentration of 30 μg protein/ml.

Membranes	NADH oxidase (μmol/min/mg protein)	
	KCl	NaCl
Wild	0.82	1.74
Nap1	1.61	1.64
Nap2	1.28	1.34

cations (5), was ineffective as a replacement for Na^+. On the other hand, the NADH oxidase activity of the mutants was completely independent of Na^+.

DISCUSSION

As expected from the close correlation between CCCP-resistant growth and the Na^+ pump, the mutants which became sensitive to CCCP were defective in the Na^+ pump. The Na^+ pump which performs CCCP-dependent H^+ uptake and CCCP-insensitive Na^+ extrusion was almost completely absent from the mutants (Figs. 1 and 2). In contrast, the mutants did retain the ability to extrude H^+. Therefore, the mutation must be specific to the Na^+ pump. The NADH oxidase of membrane fractions isolated from the mutants was altered to be independent of Na^+ (Table 2). The Na^+-requirement of NADH oxidase has also been shown in *V. costicola* (5) which was recently found to possess the respiration-coupled Na^+ pump[1]. These data strongly indicate that the Na^+-dependence of NADH oxidase represents the Na^+ pump. Since this Na^+-dependence resides exclusively at NADH:quinone oxidoreductase segment (9), it is quite likely that the Na^+ pump is localized at this segment. Although addition of TMPD which donates electrons to cytochrome c caused energization of the Na^+ pump (2), it was later found that re-reduction of oxidized TMPD by endogeneous redox energy did occur. Since the TMPD oxidase is neither dependent on Na^+ (9) nor altered in the mutants (unpublished results), the TMPD-dependent energization of the Na^+ pump seems to be derived from the formation of "TMPD-bypass" involving the Na^+-dependent site. Detailed studies on the NADH:quinone oxidoreductase as the Na^+ pump will be reported elsewhere.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Tokuda, H., and Unemoto, T. (1981) Biochem. Biophys. Res. Commun. **102,** 265-271.
2. Tokuda, H., and Unemoto, T. (1982) J. Biol. Chem. **257,** 10007-10014.
3. Mitchell, P. (1979) Eur. J. Biochem. **95,** 1-20.
4. Wikström, M., and Krab, K. (1980) Curr. Top. Bioenerg. **10,** 51-101.
5. Unemoto, T., Hayashi, M., and Hayashi, M. (1977) J. Biochem. **82,** 1389-1395.
6. Tokuda, H., Nakamura, T., and Unemoto, T. (1981) Biochemistry **20,** 4198-4203.
7. Gorini, L., and Kaufman, H. (1960) Science **131,** 604-605.
8. Tokuda, H., Sugasawa, M., and Unemoto, T. (1982) J. Biol. Chem. **257,** 788-794.
9. Unemoto, T., and Hayashi, M. (1979) J. Biochem. **85,** 1461-1467.