Release of the component of *Streptococcus faecalis* Na^+-ATPase from the membranes

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The Na^+-stimulated ATPase activity of *Streptococcus faecalis* was lost by washing the membranes with ethylenediaminetetraacetic acid (EDTA). ATPase activities of both the EDTA extract and the stripped membranes did not show any stimulation by Na^+ ions. However, the Na^+-stimulated ATPase was readily reconstituted by an incubation of these fractions combined. It was only reconstituted from the fractions prepared under the condition that the Na^+-ATPase is amplified, and not from those boiled or digested by trypsin. Thus, the component of Na^+-ATPase of this organism is capable of being released from the membranes.

Na^+-ATPase; Reconstitution; Induction; *Streptococcus faecalis*

1. INTRODUCTION

The fermentative bacterium *Streptococcus faecalis* contains a Na^+-translocating ATPase [1]. The ATPase activity is stimulated by Na^+ and Li^+, but not significantly by other ions. The Na^+-ATPase is amplified by an increase in cytoplasmic [Na^+] as a signal [2,3], and plays a central role for Na^+ circulation of this organism at alkaline pH [2]. Although the Na^+-ATPase of *S. faecalis* has not been purified, it is probably distinct from other ion-translocating ATPases as judged by (i) its resistance to vanadate as well as DCCD, and by (ii) the possibility that this enzyme exchanges Na^+ for K^+ ions [4]. In any case, the final proof of this surmise for its structure and mechanism required purification of the enzyme and functional reconstitution into proteoliposome.

We found here that the Na^+-stimulated ATPase activity of *S. faecalis* is lost when the cell membranes are washed by EDTA, and that it is readily reconstituted from EDTA extract and the stripped membranes. Thus, the peripheral protein, which constitutes the Na^+-ATPase of this organism, is released from the membranes by EDTA. The molecular structure of this component will be described in the accompanying paper [5].

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DCCD, dicyclohexylcarbodiimide

2. MATERIALS AND METHODS

2.1. *Organism and growth media*

All the experiments were conducted with a mutant of *S. faecalis* (*faecium*) defective in H^+-ATPase, designated 25D. It was isolated from AS25 [6], which was generously supplied by H. Kobayashi (Chiba University, Chiba, Japan), as one of the large colonies on the plate of complex medium at pH 9.5. Cells were grown on KTY medium or NaTY medium containing 0.4 M NaCl as described elsewhere [14].

2.2. *Preparation of cell membranes and EDTA extraction*

The cell membranes were prepared as described by Abrams [7] in the presence of 1 mM PMSF. The membranes were dialyzed overnight at 4°C in 5 liters of a buffer (1 mM Tris-HCl, 2 mM Tris-EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, 10% glycerol; pH 7.5), and then centrifuged at 100000 × *g* for 60 min. The EDTA extract recovered as clear supernatant was concentrated by the ultrafiltration (Amicon; PM 10). In order to complete the extraction, the membranes were again dialyzed overnight in the same buffer, and collected by centrifugation. The membranes were suspended in 5 mM Tris-HCl (pH 7.5) containing 2 mM $MgSO_4$, 1 mM 2-mercaptoethanol and 1 mM PMSF, and were used for reconstitution experiments as the stripped membranes.

2.3. *Reconstitution of the Na^+-ATPase*

The stripped membranes (0.15–1.0 mg) were incubated at 37°C for 30 min in 0.5 ml of a buffer (10 mM Tris-HCl, 3 mM $MgSO_4$, 1 mM PMSF and 10% glycerol; pH 7.5) with the EDTA extract (0–1.2 mg). The reconstituted membranes were subsequently collected by centrifugation at 100000 × *g* for 60 min, and suspended in the same buffer.

2.4. *Others*

The Na^+-ATPase activity was assayed as described elsewhere [3,4] with and without 25 mM NaCl in the presence of 0.5 mM DCCD. One unit indicates the amount liberating 1 μmol of P_i/min. Protein was determined as described [8] with bovine serum albumin as a standard.

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3. RESULTS

3.1. *Mg^{2+} ions are required for the Na^+-stimulated ATPase*

Fig. 1 shows the effects of EDTA and Mg^{2+} on the Na^+-stimulated ATPase activity of the membranes. The membranes were prepared from 25D, a mutant defective in H^+-ATPase, grown on NaTY medium containing 0.4 M NaCl to induce highly the Na^+-ATPase [4,9]. They were finally suspended in a buffer containing 1 mM $MgSO_4$, and were incubated at 37°C. In the ATPase assay, the membranes were diluted 40-fold into 20 mM Tris-HCl (pH 8.5), and the reaction was started by addition of 2.5 mM ATP-Mg^{2+}.

Addition of 3 mM EDTA to the membrane suspension immediately depressed the stimulation by Na^+ (Fig. 1). After 20 min about 80% of the original Na^+-ATPase activity was lost although the basal ATPase activity was little affected. By contrast, when 5 mM $MgSO_4$ was added to the membrane suspension including EDTA at 60 min, the Na^+-stimulated ATPase was instantly increased. About 90% of the original activity was recovered after 20 min (Fig. 1). The effects of EDTA and Mg^{2+} on the ATPase assays were negligible since the final concentrations of EDTA and Mg^{2+} were 0.075 mM and 0.125 mM, respectively. Thus, Mg^{2+} ions are required for the Na^+-ATPase of *S. faecalis*.

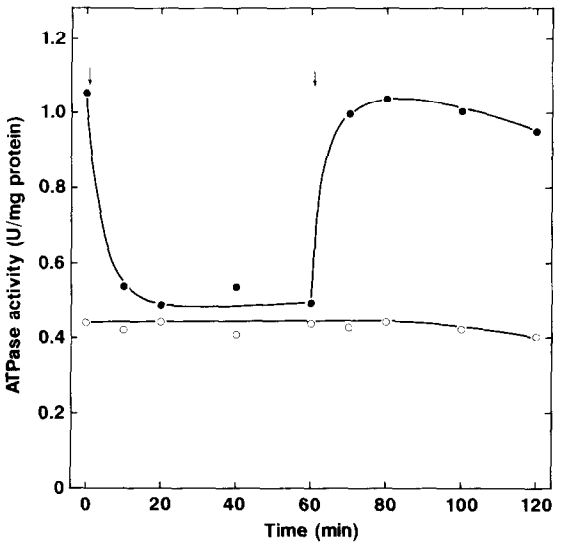

Fig. 1. Effects of EDTA and Mg^{2+} ions on the Na^+-stimulated ATPase. The membranes prepared from 25D grown on NaTY medium containing 0.4 M NaCl were suspended in a buffer (5 mM Tris-HCl, 1 mM $MgSO_4$, 1 mM 2-mercaptoethanol, 1 mM PMSF; pH 7.5), and incubated at 37°C. As indicated by the arrows, 3 mM EDTA and 5 mM $MgSO_4$ were added to the suspension at 0 min and 60 min, respectively. After diluting the membranes 40-fold into 20 mM Tris-HCl (pH 8.5), the ATPase activity was assayed with (●) and without (○) 25 mM NaCl.

3.2. *Reconstitution of the Na^+-stimulated ATPase*

In principle, the finding that the Na^+-stimulated ATPase requires Mg^{2+} ions could have several explanations. For instance, Mg^{2+} may be directly coupled with the catalytic activity of Na^+-ATPase such as a cofactor. On the other hand, as exemplified by a detachment of the F_1 portion of H^+-ATPase from the F_0 portion embedded in the membrane, chelating Mg^{2+} by EDTA often strips the peripheral proteins from the membrane. Loss of the Na^+-stimulated ATPase, alternatively, may reflect a dissociation of the component(s) of Na^+-ATPase complex. Table I shows the effect of EDTA wash of the membranes on the Na^+-stimulated ATPase. By dialysis of the membranes of 25D in a buffer containing 2 mM EDTA, about 20% of the total membrane protein was extracted, and most of the ATPase activity remained on the membranes. Only 6% of its activity was released (Table I). The ATPase activity bound to the stripped membranes did not show Na^+ stimulation, even after a much longer incubation with Mg^{2+} ions (data not shown). The ATPase activity of the EDTA extract was neither stimulated by Na^+ ions (Table I).

Laubinger and Dimroth recently discovered that the Na^+-translocating ATPase of *Propionigenium modestum* is of the F_1F_0 type [10,11]. Importantly, the F_1-ATPase released from the membranes by EDTA did not show any stimulation by Na^+ ions [10]. The Na^+-stimulated ATPase activity of this enzyme requires the direct interaction of the F_1-ATPase and the F_0 portion of F_1-depleted membranes [10]. It reminded us of the possibility that the Na^+-ATPase of *S. faecalis* also consists of a peripheral part and of the membrane-bound part. The reconstitution of the Na^+-stimulated ATPase from EDTA extract and the stripped membranes were attempted (Fig. 2). The stripped membranes were incubated with the EDTA extract in the presence of Mg^{2+}, and then the membranes were collected by centrifugation. An increase of the Na^+-stimulated ATPase activity with increasing addi-

Table I

Loss of the Na^+-stimulated ATPase by EDTA extraction

Fraction	Protein (mg)	ATPase activity (U/mg protein)		
		Without NaCl (*A*)	With 25 mM NaCl (*B*)	Na^+-stimulated (*B*) − (*A*)
Washed membranes	134.4	0.46	1.06	0.60
EDTA extract (1st)	24.8	0.14	0.13	ND
EDTA extract (2nd)	6.7	–	–	–
Stripped membranes	95.0	0.37	0.37	0.00

The mutant 25D was grown on NaTY medium containing 0.4 M NaCl, and washed membranes were prepared as described by Abrams [7] in the presence of 1 mM PMSF. The EDTA extract and the stripped membranes were prepared as described in section 2. ND, not determined.

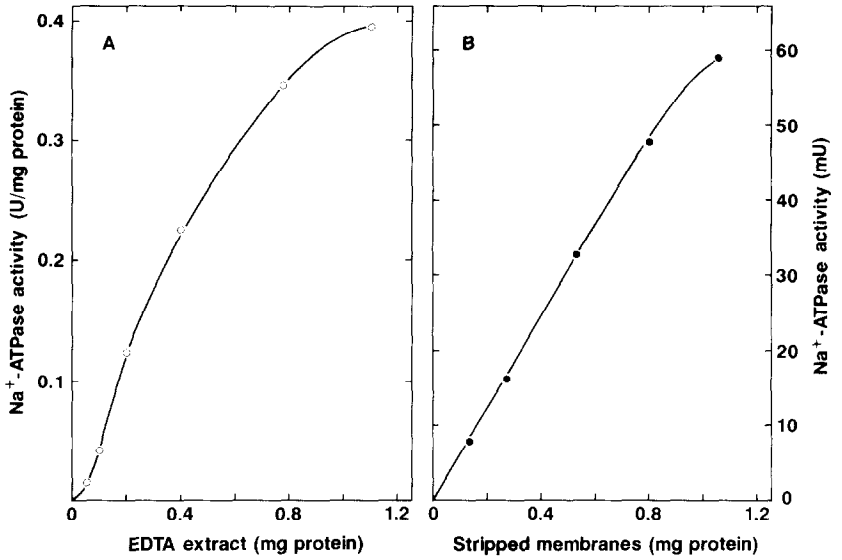

Fig. 2. Reconstitution of the Na^+-stimulated ATPase. The EDTA extract and the stripped membranes were prepared from 25D grown on NaTY medium containing 0.4 M NaCl as described in section 2. (A) Dependence on the EDTA extract. The stripped membranes (1.0 mg) were incubated with EDTA extract (0–1.2 mg) at 37°C for 30 min in 0.5 ml of a buffer (10 mM Tris-HCl, 3 mM $MgSO_4$, 1 mM PMSF and 10% glycerol; pH 7.5), and the reconstituted membranes were collected by centrifugation. The Na^+-ATPase was determined by (B) – (A) as shown in Table I. (B) Dependence on the stripped membranes. The stripped membranes (0.15–1.05 mg) were incubated with EDTA extract (1.2 mg) at 37°C for 30 min in 0.5 ml of the same buffer as described above, and the Na^+-ATPase was determined.

tions of the EDTA extract was observed (Fig. 2A), and approximately 70% of the original Na^+-stimulated ATPase activity (0.4 U/mg protein; Table I) was recovered during the reconstitution although it was not still saturated (Fig. 2A). It was not reconstituted with the EDTA extract denatured by boiling for 5 min or digested with trypsin (data not shown). The reconstitution of the Na^+-stimulated ATPase was also proportional to the amount of the stripped membranes (Fig. 2B). It was never recovered with the stripped membranes denatured by boiling (data not shown). These results suggest that a direct interaction with the soluble protein extracted by EDTA and the stripped membranes is required for the reconstitution of Na^+-stimulated ATPase.

Table II

Inducibility of the reconstituted Na^+-stimulated ATPase

Induction of the Na^+-ATPase		Na^+-ATPase activity[a] (U/mg protein)
EDTA extract	Stripped membranes	
No	No	0.02
Yes	No	0.04
No	Yes	0.06
Yes	Yes	0.38

[a] Determination by (*B*) – (*A*) as shown in Table I.

The mutant 25D was grown on KTY medium as 'uninduced' cells or NaTY medium containing 0.4 M NaCl as 'induced' cells. Preparations of EDTA extract and the stripped membranes, and the reconstitution of Na^+-ATPase were performed as described in section 2. *No* and *Yes* represent the preparation from the 'uninduced' cells and 'induced' cells, respectively.

3.3. *Inducibility of the reconstituted Na^+-stimulated ATPase*

Table II shows an inducibility of the reconstitution of Na^+-stimulated ATPase. The Na^+-ATPase of *S. faecalis* is induced when cells are grown on media rich in sodium ions [4,9]. 25D was grown on KTY medium or NaTY medium containing 0.4 M NaCl. The Na^+-ATPase activity of the original membranes was 0.07 U/mg protein from 'uninduced' cells and 0.58 U/mg protein from 'induced' cells, respectively. Even if the EDTA extract prepared from the 'induced' cells was used for the reconstitution, the Na^+-stimulated ATPase was not reconstituted with the stripped membranes prepared from the 'uninduced' cells, and vice versa. The reconstitution of Na^+-ATPase was only completed from both fractions that the Na^+-ATPase is highly induced (Table II).

The results described above suggest that the Na^+-ATPase of *S. faecalis* consists of the peripheral portion and the membrane-bound portion, and that both portions are required for Na^+ stimulation of the ATPases.

4. DISCUSSION

Although the presence of Na^+-translocating ATPase in bacteria was first reported in *S. faecalis* [1], the molecular properties of this enzyme have not been

understood well. We here found that the peripheral protein releasable from the membranes constitutes the Na^+-ATPase, and that it is required for the reconstitution of the Na^+-stimulated ATPase by a combination with the stripped membranes. Also in the Na^+-ATPase of *P. modestum*, an interaction between F_1-ATPase and the F_0 portion is required for the Na^+-stimulated ATPase activity [10]. However, we cannot attribute the catalytic portion of Na^+-ATPase of *S. faecalis* to one of these fractions, since both fractions retained the ATPase activity (Table I).

Importantly, the Na^+-ATPase of *P. modestum*, being incorporated into proteoliposome, can catalyze an ATP-dependent H^+ translocation if Na^+ is absent [12]. In the membrane vesicles of *Vibrio alginolyticus*, the presence of only one DCCD-sensitive ATPase is proposed [13]. ATP-driven movements of Na^+ and H^+ ions were sensitive to DCCD in these vesicles. Assuming that the ion selectivity of F_0 can be changed, Na^+ may be transported via the same F_0 complex [14]. Skulachev suggested that one and the same DCCD-sensitive F_1F_0-ATPase is involved in translocations of H^+ at acid pH and of Na^+ at alkaline pH [15]. Although H^+-translocating F_1F_0-ATPase exists in *S. faecalis* [16], it is unlikely to play as the machinery of Na^+-ATPase, because (i) the Na^+-ATPase activity is observed in the H^+-ATPase defective mutant [4,9], and (ii), more importantly, an antibody to the purified F_1-ATPase did not inhibit the Na^+-ATPase activity [2]. The Na^+-ATPase of *S. faecalis* is resistant to DCCD, and probably catalyzes the electroneutral Na^+/K^+ exchange rather than the electrogenic Na^+ uniport [4]. Thus, another type of the Na^+-ATPase complex, not the F_1F_0 type, exists in this organism. By polyacrylamide gel electrophoresis of the EDTA extract, we identified a component of the Na^+-ATPase of *S. faecalis*. Its properties will be described in the accompanying paper [5].

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