Some features of the *Streptococcus faecalis* Na^+-ATPase resemble those of the vacuolar-type ATPases

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In the ethylenediaminetetraacetic acid (EDTA) extract prepared from the membranes of *Streptococcus faecalis*, we found the 330-kDa protein that was coordinately increased with the induction of Na^+-ATPase. It was missed in the EDTA extract of Nak1, a mutant defective in the Na^+-ATPase, but restored in that of its revertant, Nak1R. The 330-kDa protein showed the ATP hydrolytic activity by active staining, and mainly consisted of the polypeptides of 73 kDa, 52 kDa and possibly 38 kDa. In addition, the Na^+-stimulated ATPase of the membranes was sensitive to both nitrate and *N*-ethylmaleimide, inhibitors for the vacuolar H^+-ATPase. Thus, the Na^+-ATPase of this organism has a structure similar to vacuolar H^+-ATPase.

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

1. INTRODUCTION

In the accompanying paper [1], we reported that the peripheral protein, which is releasable from the cell membranes by EDTA washing, constitutes the Na^+-translocating ATPase of *Streptococcus faecalis*. It is required for the reconstitution of the Na^+-stimulated ATPase with the stripped membranes.

In the EDTA extract, we identified a soluble 330-kDa protein as the component of Na^+-ATPase. It showed the ATP hydrolytic activity, and mainly consisted of the 73-kDa and 52-kDa polypeptides. Thus, the Na^+-ATPase of *S. faecalis*, which was remarkably inhibited by nitrate and NEM, contains a structure similar to vacuolar H^+-ATPase [2].

2. MATERIALS AND METHODS

2.1. *Organisms and growth media*

All the experiments were conducted with *S. faecalis* (*faecium*) ATCC 9790, which was generously supplied by F.M. Harold (Colorado State University, Fort Collins, Colorado, USA) and the mutants derived from it [1,3]. Organisms were grown on KTY or Na-TY medium [4], and, in some experiments, NaCl (0.5 M) or KCl (0.5 M) was included in these media. The medium pH was adjusted with maleic acid and K_2CO_3 [5].

2.2. *Preparations of cell membranes and the EDTA extract*

Cell membranes were prepared as described by Abrams [6] in the presence of 1 mM PMSF. The EDTA extract was prepared as described in the accompanying paper [1].

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide

2.3. *Gel electrophoresis and active ATPase staining*

Native PAGE in slab gels (80 × 80 × 1 mm) with 7% total and 0.2% cross-linker acrylamide was performed in a buffer system according to Laemmli [7] and run for 120 min at 15 mA. SDS-PAGE in slab gels with 12% total and 0.4% cross-linker acrylamide was also performed as described by Laemmli [7]. The polypeptides were visualized by staining with Coomassie brilliant blue R-250 or by silver staining. Marker proteins of native PAGE were thyroglobulin (670 kDa), ferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (140 kDa) and bovine serum albumin (67 kDa).

For active staining, native PAGE was performed in disc gels, and the gels were stained for ATPase activity by incubation at 37°C for 45 min in a substrate solution containing 5 mM Na_2-ATP, 5 mM $MgSO_4$, 100 mM Tris-HCl and 2.5 mM Pb-acetate, pH 8.2. To visualize the white $PbPO_4$ precipitates, gels were rinsed in H_2O and incubated for 1 min in 2% $(NH_4)_2S$ resulting in dark brown precipitates.

2.4. *Others*

The Na^+-ATPase activity was assayed as described elsewhere [1,4] with and without 25 mM NaCl in the presence of 0.5 mM DCCD. Protein was determined as described [8] with bovine serum albumin as a standard.

3. RESULTS

3.1. *Polyacrylamide gel electrophoresis of the EDTA extracts*

As described in the accompanying paper [1], the component of Na^+-ATPase is released from the membranes by EDTA wash. To identify it, native PAGE of the EDTA extract was performed (Fig. 1). First of all, in the EDTA extract prepared from 9790 grown on media poor in Na^+ ions (KTY medium or KTY containing 0.5 M KCl; pH 7.0) (Fig. 1, lanes 1 and 2), the F_1

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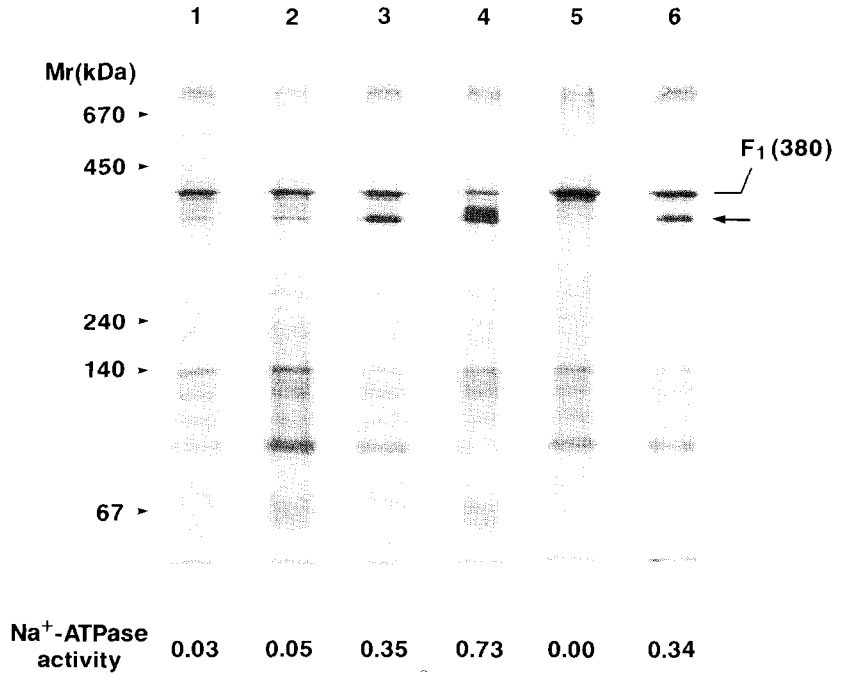

Fig. 1. Native PAGE of the EDTA extracts. The cell membranes and the EDTA extracts were prepared from 9790 and its mutants grown on various media as described in the text. About 20 μg protein of each extract was applied to the gel, and finally stained by Coomassie brilliant blue R-250 as described in section 2. Na^+-ATPase activity of the membranes was determined as described elsewhere [4,11]. Lane 1, 9790 grown on KTY; lane 2, 9790 grown on KTY plus 0.5 M KCl; lane 3, 9790 grown on NaTY plus 0.5 M NaCl; lane 4, 9790 grown on NaTY plus 0.5 M NaCl at pH 9.5; lane 5, Nak1 grown on NaTY plus 0.5 M NaCl; and lane 6, Nak1R grown on NaTY plus 0.5 M NaCl. The arrow indicates the 330-kDa protein.

portion of H^+-ATPase, whose size is about 380 kDa [9], was readily observed. The Na^+-ATPase of *S. faecalis* is highly amplified in media rich in Na^+, especially at alkaline pH [10,11]. When 9790 was grown on NaTY medium containing 0.5 M NaCl (pH 7.0) (Fig. 1, lane 3), a protein of about 330 kDa prominently appeared in the EDTA extract. In addition, when 9790 was grown at pH 9.5, the amount of the 330-kDa protein was much increased although the band became broad and slightly tailed (Fig. 1, lane 4). It was also slightly detected in both lanes 1 and 2 of Fig. 1. Considering both the PAGE profiles and the Na^+-ATPase activities of the original membranes prepared from these cells, the emergence of a 330-kDa protein in the extract just corresponds to amplification of Na^+-ATPase. Nak1 is a single mutant only defective in the Na^+-ATPase [3]. Importantly, only the 330-kDa protein was missing in the EDTA extract of Nak1 (Fig. 1, lane 5), but it was restored in that of the revertant of Nak1, Nak1R (Fig. 1, lane 6). The Na^+-ATPase activity was missing in the membranes of Nak1, but restored in those of Nak1R. The reconstitution of the Na^+-stimulated ATPase was also examined from these EDTA extracts and the stripped membranes of 9790 in which the Na^+-ATPase was induced. The Na^+-ATPase was reconstituted with the extracts containing the 330-kDa protein, but not with those of Nak1 (data not shown). Moreover, we performed 12% PAGE of the EDTA extracts as shown in Fig. 1, but could not find any remarkable differences among them except for the 330-kDa protein. These results suggest that the 330-kDa protein is the component of Na^+-ATPase released from the membranes.

To examine the possibility that this component of 330 kDa is the catalytic portion of Na^+-ATPase, active ATPase staining of the EDTA extract was performed (Fig. 2). The EDTA extracts were prepared from 25D, a mutant defective in the H^+-ATPase [1] grown on KTY medium or NaTY medium containing 0.4 M NaCl. In contrast with the limited amount of the F_1-ATPase in both extracts (Fig. 2A), the 330-kDa protein was prominently increased in the EDTA extract prepared from the cell in which the Na^+-ATPase was induced (Fig. 2A, lane 2). By active staining, only two bands were detected in the extract of 25D in which the Na^+-ATPase was limited (Fig. 2B, lane 1). The molecular size of these two bands coincided with the 330-kDa protein and the F_1-ATPase. In the EDTA extract in which the Na^+-ATPase was highly induced, the staining of the 330-kDa protein was remarkably increased (Fig. 2B, lane 2). The ATPase staining on the 330-kDa protein was also observed in the extract of 9790, but little observed in that of Nak1. It was restored in the extract of Nak1R (data not shown). Thus, the 330-kDa component of Na^+-ATPase retains the ATP hydrolytic activity.

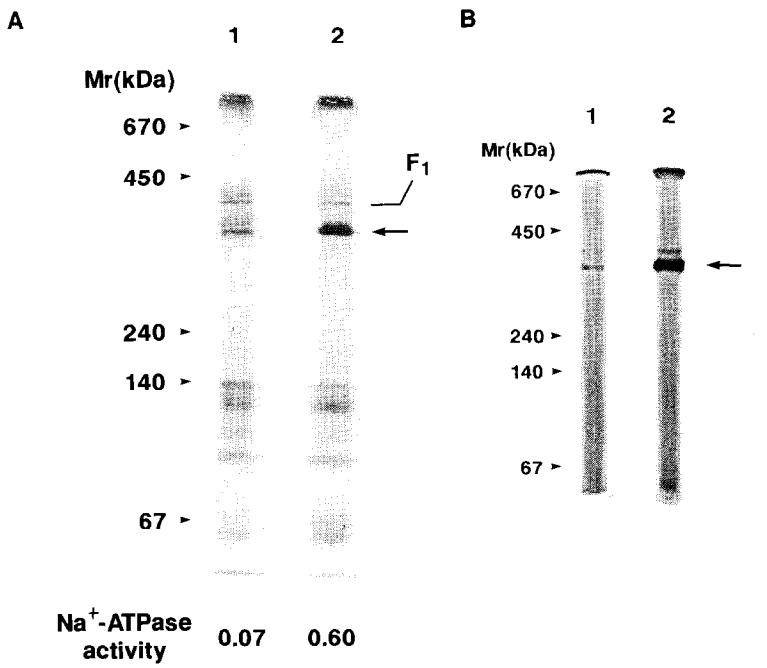

Fig. 2. Active ATPase staining of the EDTA extract. The cell membranes and the EDTA extracts were prepared from 25D grown on KTY medium (lane 1) or NaTY medium containing 0.4 M NaCl (lane 2). (A) Native PAGE of the EDTA extracts. About 20 μg protein of each extract was applied. Na^+-ATPase activity of the membranes was determined as described elsewhere [4,11]. (B) Active ATPase staining. About 340 μg protein of the extract was applied on the disc gel, and active staining was performed as described in section 2. The arrow indicates the 330-kDa protein.

3.2. *SDS polyacrylamide gel electrophoresis of the EDTA extracts*

Fig. 3 shows SDS-PAGE of the EDTA extracts (Fig. 3). The EDTA extracts were prepared from the cells grown on media in which Na^+-ATPase is highly induced. The polypeptides of about 73 kDa, 52 kDa and 38 kDa distinctly observed in the extract of 9790 were missing in that of Nak1 (Fig. 3A, lanes 1 and 3). However, they were restored in the extract of Nak1R (Fig. 3A, lane 4). The amounts of these polypeptides

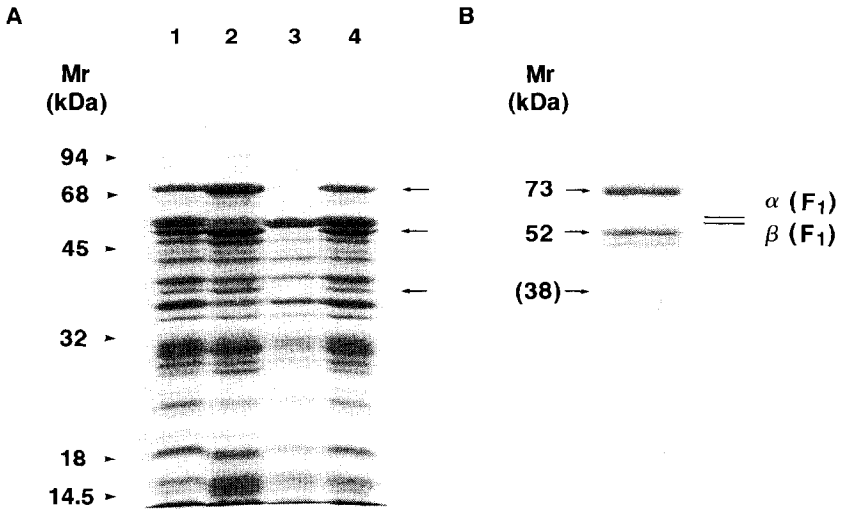

Fig. 3. SDS-PAGE of the EDTA extracts and the 330-kDa protein. (A) SDS-PAGE of the EDTA extracts. About 20 μg of the same sample as shown in Fig. 1 was applied, except for 15 μg of the extract of Nak1, and stained by Coomassie brilliant blue R-250 as described in section 2. Lane 1, 9790 grown on NaTY plus 0.5 M NaCl; lane 2, 9790 grown on NaTY plus 0.5 M NaCl at pH 9.5; lane 3, Nak1 grown on NaTY plus 0.5 M NaCl; and lane 4, Nak1R grown on NaTY plus 0.5 M NaCl. The arrows indicate the polypeptides that coordinate the induction of Na^+-ATPase. (B) SDS-PAGE of the 330-kDa protein. After native PAGE in a slab gel, the band of 330-kDa protein was cut, and extracted from the gel electrophoretically. Three μg was applied, and stained by CBB. As a reference, the bands of both α- and β-subunits of the H^+-ATPase of *S. faecalis* were illustrated. The 38-kDa polypeptides parenthesized were only detected by silver staining.

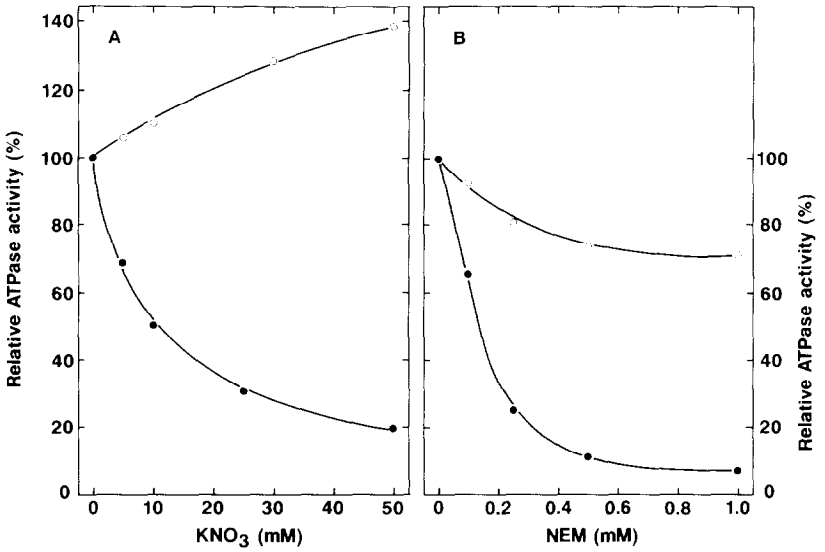

Fig. 4. Effects of nitrate and NEM on the ATPases of *S. faecalis*. The membranes were prepared from 25D grown on NaTY medium containing 0.4 M NaCl for the Na^+-ATPase or from 9790 grown on KTY medium for the H^+-ATPase. Assays were performed at pH 8.5 for the Na^+-ATPase (●) and at pH 7.0 for the H^+-ATPase (○), respectively. Nitrate (A) and NEM (B) were added 10 min prior to initiating the reaction, respectively. The Na^+-ATPase activity was determined as described elsewhere [4,11]. The activity represented as 100% was 0.6 μmol/min per mg protein of the Na^+-ATPase and 0.8 μmol/min per mg protein of the H^+-ATPase, respectively.

were all increased in the extract of 9790 grown at pH 9.5 (Fig. 3A, lane 2). When the EDTA extracts were prepared from 9790 or 25D grown on media in which induction of the Na^+-ATPase was limited (Fig. 1, lanes 1 and 2; Fig. 2, lane 1), these polypeptides were only slightly detected (data not shown). Thus, at least these polypeptides correspond to induction of the Na^+-ATPase of *S. faecalis*.

To know if these polypeptides are the subunits of 330-kDa ATPase of the EDTA extract, the 330-kDa protein was extracted from the gel after electrophoresis, and analyzed by SDS-PAGE. Both 73-kDa and 52-kDa polypeptides were detected in this fraction. It is unknown whether a faint band observed below the 52-kDa band is another subunit or a proteolytic artifact of the 73-kDa or 52-kDa subunit. In addition, the 38-kDa polypeptide observed in the EDTA extracts (Fig. 3A) was also able to be detected with other minor polypeptides by silver staining (data not shown). Considering the molecular sizes of α- and β-subunit of F_1-ATPase as illustrated in Fig. 3B, the subunit composition of the 330-kDa component of Na^+-ATPase obviously differs from that of the F_1-type ATPase [2,9].

3.3. *Sensitivity of the Na^+-ATPase to nitrate and NEM*

Finally the effects of nitrate and *N*-ethylmaleimide on the Na^+-ATPase of the membranes was examined (Fig. 4). Both reagents are known to be the specific inhibitors for H^+-ATPase of vacuolar type and archaebacterial type [2,12,13]. The Na^+-stimulated ATPase was remarkably inhibited by nitrate (K_i: about 10 mM) (Fig. 4A) and NEM (K_i: about 0.15 mM) (Fig. 4B). On the other hand, the F_1-ATPase activity of *S. faecalis* was activated by nitrate and only slightly inhibited by NEM (Figs 4A and B).

These results suggest that the Na^+-ATPase of *S. faecalis* contains a structure similar to the vacuolar (archaebacterial) H^+-ATPase [2,12,13].

4. DISCUSSION

Although the existence of Na^+-translocating ATPase is expected in some bacteria, an electrogenic Na^+-ATPase of *Propionigenium modestum* has only been purified [14,15]. It is very similar to the H^+-ATPase of *Escherichia coli* in both subunit composition and sensitivity to DCCD. A direct interaction between the F_1 part and F_0 part of the enzyme is required for the Na^+-stimulated ATPase activity [14].

Streptococcus faecalis also contains an Na^+-translocating ATPase [10], but the enzyme is distinct from the Na^+-ATPase of *P. modestum*, since (i) it is resistant to DCCD, and (ii) it probably catalyzes an electroneutral Na^+/K^+ exchange rather than an electrogenic Na^+ uniport [4]. Thus, another type of the Na^+-ATPase exists in this organism.

We identified here the 330-kDa protein in the EDTA extract as the component of Na^+-ATPase of *S. faecalis*. It is probably the catalytic portion of Na^+-ATPase because of retaining the ATP hydrolytic activity (Fig. 2B). By SDS-PAGE it was multimeric and mainly consisted of the two subunits of 73 kDa and 53 kDa. In addition, Na^+-ATPase activity of the membranes was sensitive to nitrate and NEM, inhibitors for the

vacuolar H^+-ATPase [2]. These results suggest the similarity of the Na^+-ATPase of *S. faecalis* to the V-type ATPase, but not the F-type ATPase [2]. Although H^+-ATPases similar to the V-type ATPase were discovered in several archaebacteria [12,13], this is the first report indicating the presence of the V-like Na^+-ATPase in bacteria. We are now investigating the immunological cross-reactivity between the 330-kDa protein and the V-type ATPase. Purification of the 330-kDa protein and gene cloning by its antibody are also in progress.

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