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Export of α-Amylase by *Bacillus amyloliquefaciens* Requires Proton Motive Force

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The secretion of protein directly into the extracellular medium by *Bacillus amyloliquefaciens*, a gram-positive bacterium, was shown to be dependent on proton motive force. When the electrochemical membrane potential gradient of protons was dissipated either by uncouplers or by valinomycin in combination with K^+, a precursor form of α-amylase accumulated on the cellular membrane. The proton motive force could be dissipated without altering the intracellular level of ATP, indicating that the observed inhibition of export was not the result of decreased ATP concentration.

Proton motive force is essential to the translocation of polypeptides through membranes in some but not all systems. Export of protein in *Escherichia coli*, a gram-bacteria bacterium, requires an electrochemical membrane potential, as does import of protein to the inner compartments of mitochondria. However, passage of protein through the membrane of the rough endoplasmic reticulum during secretion is independent of energy (L. L. Randall, Methods Enzymol., in press). Whereas the lumen of the endoplasmic reticulum is bounded by a single membrane, gram-negative bacteria and mitochondria are each surrounded by two membranes. Thus, the energy requirement in the latter two systems might be related to that common structural feature. If this were the case, then a gram-positive bacterium, bounded by a single membrane analogous to the rough endoplasmic reticulum, would not require energy. Therefore, we began investigations of the energetics of export in *Bacillus amyloliquefaciens*. We conclude that the energy requirement seen in mitochondria and *E. coli* is not likely to be related to the presence of a double membrane, because, as we report here, *B. amyloliquefaciens* requires proton motive force to secrete α-amylase through a single membrane.

MATERIALS AND METHODS

Chemicals. [^{35}S]methionine (approximately 1,000 Ci/mmol) was purchased from New England Nuclear Corp., and *Staphylococcus aureaus* V8 protease was obtained from Miles Laboratories, Inc. Chloramphenicol, phenylmethylsulfonyl fluoride (PMSF), lysozyme, firefly lantern extract, valinomycin, and α-amylase were purchased from Sigma Chemical Co. SF 6847 was a gift from Evert Bakker.

Strains and growth conditions. *B. amyloliquefaciens* SYC 5085 was grown at 37°C with shaking in M9 minimal medium (9) containing 18 amino acids (all except methionine and cysteine) at 1 mM and 33 mM glucose.

Pulse-labeling. Exponentially growing cells were pulse-labeled at a density of 4×10^8 cells per ml. The cell culture (1 ml) was labeled for 30 s with 40 μCi of [^{35}S]methionine (pulse-label), and then incorporation of label was terminated by the addition of unlabeled methionine to a concentration of 0.1 mM. The culture was incubated for an additional 60 s (nonradioactive chase) and then poured onto 0.5 ml of frozen 10 mM Tris-acetate (pH 7.8)–0.1 mM PMSF. In experiments involving labeling in the presence of uncouplers, the cultures were incubated in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) (10) for 30 s immediately before the addition of radioactive methionine. When the membrane was depolarized by valinomycin treatment, the cells were incubated for 10 min in the presence of valinomycin at a final concentration of 30 nmol/mg (dry weight) of cells. Potassium chloride or sodium chloride was then added, and the cells were incubated for 1 min immediately before labeling. Because treatment of cells with uncoupler or valinomycin-salt inhibited incorporation of [^{35}S]methionine by 70%, the labeling period was 90 s, and the subsequent incubation with nonradioactive methionine was 180 s.

Cellular fractionation. All manipulations were performed at 4°C unless otherwise indicated, and all buffers contained 0.1 mM PMSF. Pulse-labeled cells were separated from extracellular proteins by centrifugation at 15,600 × *g* for 10 min (Eppendorf centrifuge, model 5412). The supernatant was saved, and the cells were suspended in 0.5 ml of 10 mM Tris-acetate (pH 7.8) and centrifuged again to remove any residual extracellular α-amylase. The supernatants from these two centrifugations were combined as the extracellular fraction. The cells were suspended in 0.5 ml of 10 mM Tris-acetate (pH 7.8) and incubated on ice for 30 min in the presence of 400 μg of lysozyme per ml. The cells were broken by ultrasonic disruption (15 s in Eppendorf tubes by using a Tekmar sonicator with a water-jacketed cup horn). This suspension was used as the source of cell-associated protein or was further fractionated by centrifugation at 223,000 × *g* for 60 min in a Beckman 42.2 rotor. The supernatant was taken as the soluble fraction. The pellet was suspended in 0.5% sodium dodecyl sulfate (SDS)–10 mM Tris-acetate (pH 7.8) and represents the membrane fraction.

Immunoprecipitation. All buffers except the gel electrophoresis sample buffer contained 0.1 mM PMSF. Samples to be immunoprecipitated were brought to 0.5% in SDS and incubated at 55°C for 10 min. Triton X-100 was added to 2%, the samples were clarified by centrifugation at 15,600 × *g* for 10 min, and the supernatant was added to a tube containing antiserum raised against α-amylase. Nonradioactive α-amylase was added to act as carrier protein. Samples were incubated on ice for 15 min and then frozen. After the samples were thawed, the following procedure was performed to wash the immunoprecipitates: centrifugation to pellet the precipitate; suspension in 0.2 ml of 0.15 M

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NaCl–10 mM Tris-acetate (pH 7.8)–0.5% Triton X-100–5 mM EDTA; centrifugation and suspension of the pellet in 0.2 ml of the above solution with an NaCl concentration of 0.5 M. This wash was followed by a wash in the 0.15 M NaCl solution, and that was followed by one wash with 10 mM Tris-acetate (pH 7.8). The pellet of the final wash was suspended in 20 μl of sample buffer for gel electrophoresis.

Gel electrophoresis and autoradiography. SDS-polyacrylamide gel electrophoresis and autoradiography were performed as previously described (11).

Limited proteolysis. Peptide maps were generated by limited proteolysis (2). The protein to be digested was prepared by excising a band from a polyacrylamide gel after autoradiography and soaking the gel slice in sample buffer for 30 min. Limited proteolysis was performed in a 15% acrylamide gel by first electrophoresing 20 μl of sample buffer containing 0.05 μg of *S. aureus* V8 protease until the protease just entered the stacking gel. The rehydrated gel piece was then placed in the sample well and electrophoresed until the tracking dye was about 1 cm above the separation gel, and electrophoresis was stopped for 45 min to allow proteolysis to proceed. Electrophoresis was then continued as usual.

Determination of ATP level. Cells were grown in minimal medium as given above to a density of 4×10^8 cells per ml. Cells (0.5 ml) were added to 0.5 ml of ice-cold 2 M perchloric acid. The samples were centrifuged to remove acid-insoluble material, and the supernatants were neutralized with 2 M KOH and then frozen in an ice-salt bath. The samples were thawed, and the precipitated potassium perchlorate was removed by centrifugation. A portion of the supernatant (0.15 ml) was added to 0.9 ml of 20 mM glycylglycine–5 mM sodium arsenate–4 mM $MgSO_4$ (pH 8.0). For determination of ATP concentrations, 10 μl of firefly lantern extract was added, and fluorescence was determined with a Beckman LS-230 scintillation counter (7). When ATP determinations were to be done on cells treated with 20 μM CCCP, the uncoupler was added 4 min before the addition of the perchloric acid.

RESULTS

B. amyloliquefaciens, a gram-positive bacterium, secretes protein directly into the extracellular medium. The appearance of newly synthesized protein in the extracellular medium can be inhibited by treating the cells with the uncoupler CCCP to dissipate proton motive force before labeling with [^{35}S]methionine (Fig. 1). Treatment of the cells with a different uncoupler, SF 6847, produced a similar inhibition of export (Fig. 1). Collapse of the electrochemical membrane potential can result in depletion of internal ATP, because the proton-translocating ATPase will hydrolyze ATP in a futile attempt to restore proton motive force. Therefore, it was necessary to demonstrate directly that the ATP levels in the cell were unperturbed under conditions in which export could be inhibited by treatment with the uncouplers. When 33 mM glucose was present during the experiment, the ATP level in cells incubated with 20 μM CCCP (10.2 nmol of ATP per mg [dry weight]) was essentially the same as in control cells (10.1 nmol of ATP per mg [dry weight]). See Materials and Methods for details. When cells were treated with 20 μM CCCP in the absence of a carbon source, the ATP level fell to 30% of that of the control. Therefore, all experiments designed to inhibit export specifically by dissipation of proton motive force were performed in the presence of 33 mM glucose.

One of the best characterized among the proteins exported by *B. amyloliquefaciens* is α-amylase (14). Analysis of

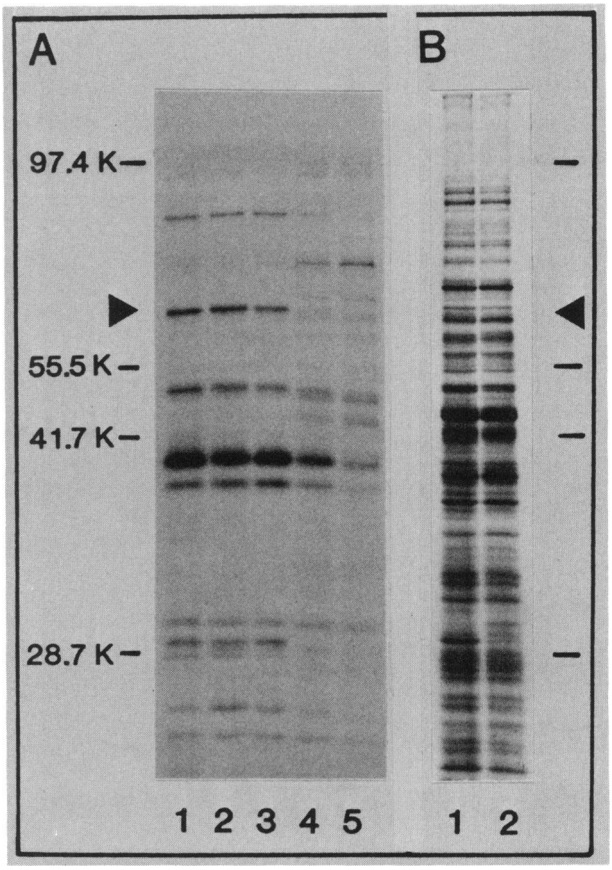

FIG. 1. Effect of uncouplers on protein export. Cultures were incubated in the presence of uncouplers at the concentrations noted below for 2 min and thereafter labeled with [^{35}S]methionine for 15 min. The samples were fractionated into extracellular and cell-associated protein as described in Materials and Methods. Protein was precipitated with 5% trichloroacetic acid. Nonradioactive lysozyme was added to the extracellular fractions to serve as carrier protein. The samples were analyzed by SDS-polyacrylamide (10%) gel and autoradiography. (A) Extracellular proteins. Each lane contains material from 6.25×10^7 cell equivalents. Lanes: 1, no addition of uncoupler; 2, 5 μM CCCP; 3, 10 μM CCCP; 4, 50 μM CCCP; 5, 40 μM SF 6847. (B) Cell-associated proteins. Each lane contains material from 4×10^6 cell equivalents. Lanes: 1, no addition of uncoupler; 2, 50 μM CCCP. Molecular weight markers were as follows: phosphorylase B, 97.4 kilodaltons (kDa); glutamic dehydrogenase, 55.5 kDa; actin, 41.7 kDa; carbonic anhydrase, 28.7 kDa. The arrowheads indicate the position of α-amylase.

cultures pulse-labeled after treatment with CCCP revealed that the disappearance of α-amylase from the extracellular medium (Fig. 1 arrow) was accompanied by the appearance of a higher-molecular-weight form of α-amylase that remained cell associated. Most of the higher-molecular-weight form was recovered in the membrane pellet when disrupted cells were subjected to high-speed centrifugation (Fig. 2).

The relationship of the higher-molecular-weight form to authentic α-amylase was investigated by analysis of peptide patterns derived from limited proteolysis of the two polypeptides (Fig. 3). The patterns were very similar, the difference being that a peptide present in the digest of the authentic mature protein was replaced by a peptide with a molecular weight approximately 4,000 larger in the digest of the higher-molecular-weight form. This peptide most likely is derived from the amino terminus and contains the uncleaved signal sequence. The higher-molecular-weight

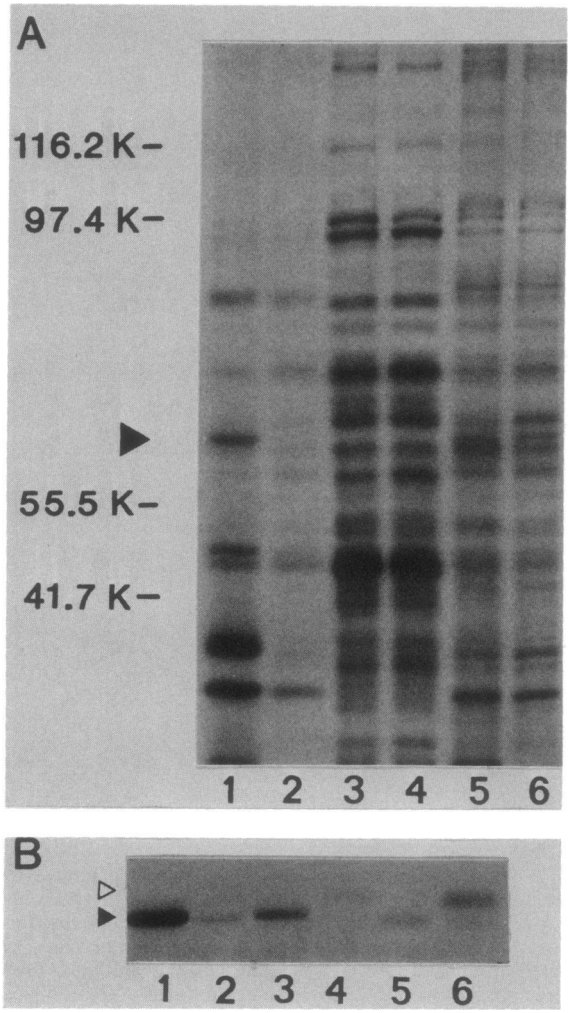

FIG. 2. Localization of precursor α-amylase. Cells were pulse-labeled with [^{35}S]methionine in the absence (lanes 1, 3, and 5) and the presence (lanes 2, 4, and 6) of 20 μM CCCP and then incubated with nonradioactive methionine as described in Materials and Methods. Cells were fractionated into extracellular (lanes 1 and 2), soluble (lanes 3 and 4), and membrane (lanes 5 and 6) fractions, and α-amylase was immunoprecipitated from each fraction. The polypeptides were analyzed by SDS-polyacrylamide (10%) gel electrophoresis and autoradiography. (A) Total protein patterns. Lanes 1 and 2 contain material from 2×10^8 cell equivalents; lanes 3 through 6 contain material from 4×10^6 cell equivalents. (B) Immunoprecipitate of α-amylase. Samples were those shown in panel A, except that all lanes contain material from 2×10^8 cell equivalents. Molecular weight markers were as given in Fig. 1 with the addition of β-galactosidase, 116.2 kDa. The closed arrowheads indicate the position of mature α-amylase, and the open arrowhead indicates the position of precursor.

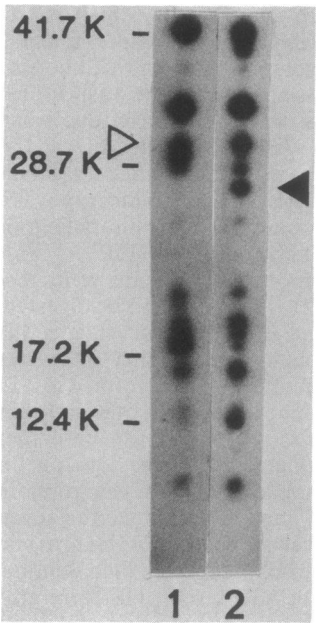

FIG. 3. Limited proteolysis of precursor and mature forms of α-amylase. Cells labeled for 5 min with [^{35}S]methionine in the absence and presence of 5 μM SF 6847 were immunoprecipitated and analyzed by SDS-polyacrylamide (10%) gel electrophoresis. After autoradiography, gel pieces containing precursor and mature protein were cut out and subjected to limited proteolysis as described in Materials and Methods followed by autoradiography. Lane 1: Precursor α-amylase from SF 6847-treated cells. Lane 2: Mature α-amylase from control cells. The open arrowhead indicates a peptide in the precursor protein that is shifted to a position higher than the position of the corresponding peptide (closed arrowhead) in the mature protein. Molecular weight markers were as in Fig. 1 and 2 with the addition of myoglobin, 17.2 kDa, and cytochrome *c*, 12.4 kDa.

species, accumulated in the presence of CCCP, was slowly converted to the mature form during a subsequent incubation, thus demonstrating that it is a precursor of α-amylase (Fig. 4).

To eliminate the possibility that the uncouplers interfered with export through an action other than dissipation of proton motive force (for example, CCCP reacts with cysteine [6]), the effect of valinomycin and K^+ was investigated. When valinomycin-treated cells were pulse-labeled after the addition of potassium chloride, the precursor form of α-amylase was accumulated, and it remained cell associated during the chase (Fig. 5A). As a control, valinomycin-treated cells were pulse-labeled after the addition of sodium chloride. The only form of α-amylase detected in these cells was the mature form which was cell associated during the pulse (Fig. 5A) and released into the extracellular medium during the subsequent chase (Fig. 5B). The maturation and export

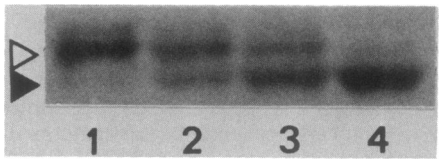

FIG. 4. Maturation of precursor protein. Cells were pulse-labeled for 90 s in the presence of 20 μM CCCP and thereafter incubated with an excess of nonradioactive methionine (chase) for times as given. Cells were separated from the extracellular fraction as described in Materials and Methods, and α-amylase was immunoprecipitated from 2×10^8 cell equivalents of cell-associated protein. Lanes: 1, 20-s chase; 2, 2-min chase; 3, 4-min chase; 4, 9-min chase. The open and closed arrowheads indicate the positions of precursor and mature α-amylase, respectively.

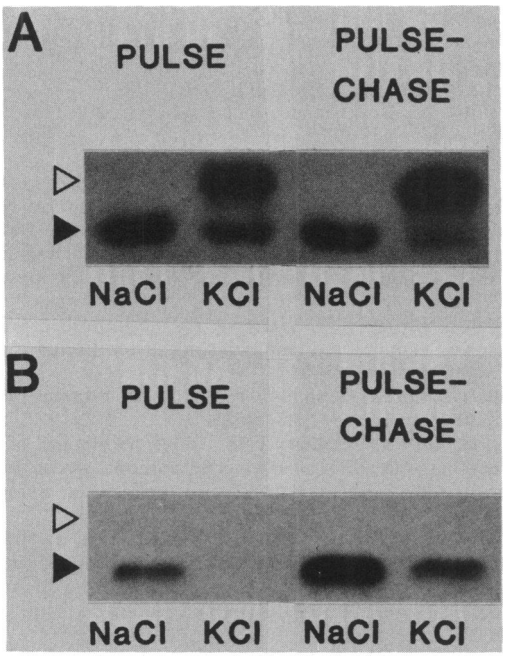

FIG. 5. Effect of valinomycin-KCl. Sodium chloride (250 mM) or potassium chloride (250 mM) was added to valinomycin-treated cells. The cultures were labeled for 90 s (sample withdrawn as pulse), then incubated with an excess of nonradioactive methionine for 180 s (pulse-chase), and fractionated into extracellular and cell-associated proteins, and the fractions were subjected to immunoprecipitation. The immunoprecipitates were analyzed by SDS-polyacrylamide (10%) gel electrophoresis and autoradiography. (A) Cell-associated protein. (B) Extracellular protein. The open and closed arrowheads indicate the positions of precursor and mature α-amylase, respectively.

of α-amylase was unperturbed in cells treated with valinomycin in the absence of added salt. Export was also normal in cells with only potassium chloride or sodium chloride (data not shown). Thus, the observed accumulation of the cell-associated precursor form cannot be a side effect of salt or valinomycin but resulted from the action of valinomycin as a K^+ ionophore that dissipates the electrical membrane potential.

DISCUSSION

Mitochondria and *E. coli* have features in common: each is surrounded by an envelope comprising two membranes, each generates an electrochemical gradient of protons across its inner membrane, and each requires this form of energy for translocation of protein during biogenesis. Proton motive force is generated in both systems with the same polarity (inside negative), but protein is moved in opposite directions; thus, mitochrondria translocate protein from the outside to the inside, whereas *E. coli* move protein from the inside out. Schatz and Butow (12) proposed that energy is required to stabilize zones of contact between the inner and outer membranes and that these structures are critical for translocation. This proposal finds support in the observation that proteins localized to the intermembrane space in mitochrondria are proteolytically processed by a soluble enzyme residing in the matrix (3, 4). Thus, at some point during transit, the amino terminus of the protein must penetrate both membranes. Contact points between cytoplasmic and outer membranes in *E. coli*, Bayer's zones of adhesion, have been implicated in protein export by the demonstration that newly synthesized porin appeared in the outer membrane at approximately 200 to 400 discrete sites distributed uniformly over the cell surface (13).

If maintenance of zones of contact between membranes were the explanation for the requirement for energy during protein translocation, then gram-positive bacteria, surrounded by a single membrane, would not display a need for proton motive force. However, dissipation of proton motive force in gram-positive bacteria does inhibit export. Tweten and Iandolo (15) used the uncouplers CCCP and 2,4-dinitrophenol to inhibit the proteolytic conversion of the precursor of enterotoxin B to the mature form in *S. aureus*. These authors suggested that proton motive force is required in export; however, they did not rule out secondary effects of the uncouplers such as depletion of cytoplasmic ATP. Here we have shown that dissipation of proton motive force in *B. amyloliquefaciens* results in the accumulation of the unprocessed precursor form of α-amylase. The precursor remains associated with the cytoplasmic membrane, and it seems likely that the block is in translocation across that membrane. The observed effect of uncouplers on export was not the result of the depletion of cytoplasmic ATP levels, because direct determination demonstrated that the internal concentration of ATP was unaffected by the addition of uncouplers under the conditions used. To eliminate other unexpected side effects of the inhibitory compounds, valinomycin was used. Valinomycin inhibited export only if K^+ was present. No effect was seen with the addition of valinomycin alone, of valinomycin plus Na^+, or of either K^+ or Na^+ in the absence of valinomycin. Thus, the inhibition was the result of valinomycin acting as a K^+ ionophore. As K^+ flows into the cell, not only is the membrane potential dissipated, but the cellular volume increases. The swelling of the cell might indirectly affect membrane-associated phenomena by stretching the membrane. Therefore, we also examined the effect of uncouplers. Uncouplers cause the opposite change in cellular volume; proton motive force is necessary to maintain cytoplasmic pools of ions; dissipation of the energy thus results in efflux of ions and cell shrinkage. Because both valinomycin-K^+ and uncouplers inhibit export while they have opposite effects on cellular volume, we conclude that the inhibition is the result of the dissipation of proton motive force.

In *E. coli* it has been shown that both components of proton motive force, the pH gradient and electrical membrane potential, function equally well in the export of β-lactamase (1). It seems likely that the total proton motive force is utilized by *B. amyloliquefaciens*, although this remains to be demonstrated. In addition, it is not yet clear whether the requirement for energy in export is absolute. There are reports that both gram-negative and gram-positive bacteria can grow in the absence of proton motive force (5, 8); thus, the requirement may be kinetic.

The precise role of proton motive force in the translocation of protein through membranes remains undefined. Assuming that the role of energy is the same in gram-negative and gram-positive bacteria, we can eliminate the possibility that proton motive force is necessary to maintain zones of contact between the inner and outer membranes in gram-negative bacteria. More data are needed to distinguish between an indirect role, such as a requirement for an energized membrane to keep components of the export apparatus in an active conformation, and a direct role

whereby the movement of H^+ would be directly coupled to the translocation of the exported protein.

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