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Proton Motive Force Involved in Protein Transport Across the Outer Membrane of *Aeromonas salmonicida*

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Many Gram-negative bacteria export proteins to the exterior. Some of these proteins are first secreted into the periplasm and then cross the outer membrane in a separate step. The source of energy required for the translocation is unknown. Export of the extracellular protein proaerolysin from the periplasm through the outer membrane of *Aeromonas salmonicida* is inhibited by a proton ionophore and by low extracellular *p*H. One possible explanation of these results is that a proton gradient across the outer membrane is required for export.

THE OUTER MEMBRANE OF GRAM-negative bacteria is thought to be a rather inert structure that allows the free movement of small molecules through holes created by porins but forms a barrier to large molecules like proteins (*1*). Yet this membrane must be crossed by proteins that are released into the culture supernatant by the Gram-negative species capable of export. The mechanisms used to accomplish this have received little attention, in part because *Escherichia coli* does not appear to have a general extracellular secretory system. Release of proteins by this species requires the participation of other gene products or results from rupture of the outer membrane (*2*).

The limited evidence available from research with other species suggests that two general pathways are followed during protein export (*3*). In the first, the two membranes appear to be crossed simultaneously, at zones of adhesion. This pathway would seem to be the least complicated and most direct, yet of those proteins studied, only exotoxin A of *Pseudomonas aeruginosa* is said to be released in this way (*4*). Other proteins traverse the inner and outer membranes in separate steps, entering the periplasm during transit. Two examples are proaerolysin, the precursor of the hemolytic toxin of *Aeromonas hydrophila* (*5*), and choleragen of *Vibrio cholerae* (*6*). Like outer membrane and periplasmic proteins, they first cross the inner membrane, and their signal sequences are removed before they are released into the periplasm. From there they somehow cross the outer membrane and appear in the culture supernatant as water-soluble proteins. How they get through the outer membrane is not understood. Although there are large concentration gradients between the periplasm and the exterior for both proaerolysin in *A. hydrophila* and choleragen in *V. cholerae*, it is hard to imagine how the concentration of the proteins could be enough to drive them across the outer membrane. Nor can transfer be a consequence of covalent modification, as the periplasmic forms of both proteins are functionally and physically indistinguishable from the forms isolated from the culture supernatants.

The secretion of proteins across the inner bacterial membrane requires an electrochemical gradient (*7*). Membrane potentials and chemical gradients of protons are not usually considered as potential driving forces for protein export, however, because the holes formed by porins in the outer membrane are believed to allow free movement of small molecules. Nevertheless, there is some evidence that an appreciable electrical potential can exist across the outer membrane of *E. coli* because of the large concentration of negative charges in the periplasm, and it is estimated that the *p*H of the periplasm should be significantly lower than the *p*H of the exterior medium (*8*).

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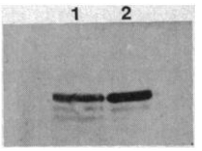

Fig. 1. Molecular form of intracellular aerolysin. *A. salmonicida* AS440 (ATCC 14174) containing the plasmid pKW2 (*10*) was grown at 27°C to an optical density at 600 nm (OD_{600}) of 2.4 in LB. The cells were centrifuged and immediately resuspended in the sample buffer used in sodium dodecyl sulfate electrophoresis (*15*) and boiled. After electrophoresis, the proteins were transferred to nitrocellulose and immunoblotted with a mouse monoclonal antibody to aerolysin (*16*): lane 1, total cell contents; lane 2, purified proaerolysin. Identical results were obtained with rabbit polyclonal antibody to aerolysin.

Recently we cloned the structural gene for proaerolysin into *A. salmonicida* (*9*). The protoxin is exported from the bacteria to the culture supernatant, and during export it is possible to measure a pool of cell-associated proaerolysin. Most of this pool can be recovered by osmotic shock, indicating that it is periplasmic, although it may be weakly associated with one of the membranes. When the bacteria are transferred to fresh medium containing chloramphenicol (to prevent the synthesis of new protein), time-dependent release of the protoxin results. Here we use this system to study the mechanism of transfer of proaerolysin across the outer membrane of *A. salmonicida*.

In the first experiment, bacteria were grown in LB medium at *p*H 7.5. The results in Fig. 1 show that no preprotoxin could be detected in the cells, indicating that the signal sequence had been removed. This is further evidence that most of the proaerolysin found in these cells has crossed the inner membrane. When the cells were transferred to fresh media containing chloramphenicol, the shockable pool of proaerolysin was rapidly depleted, resulting in the appearance of the protoxin in the medium (Fig. 2) as we have found earlier. The results in Fig. 2 also show that release of proaerolysin was virtually completely inhibited by the presence of the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) in the new media. The most obvious interpretation of this result is that export of proaerolysin across the outer membrane is somehow coupled to the electrochemical gradient across the inner membrane and that the gradient is dissipated by CCCP. Such a system, which involves TonB and other proteins, is said to be required for the import of colicins and some nutrients across the outer membrane of *E. coli* (*10*).

Another possibility is that CCCP abolishes a proton gradient across the outer membrane that is required for proaerolysin export. If this were the case, then reducing the size of the gradient by lowering the *p*H of

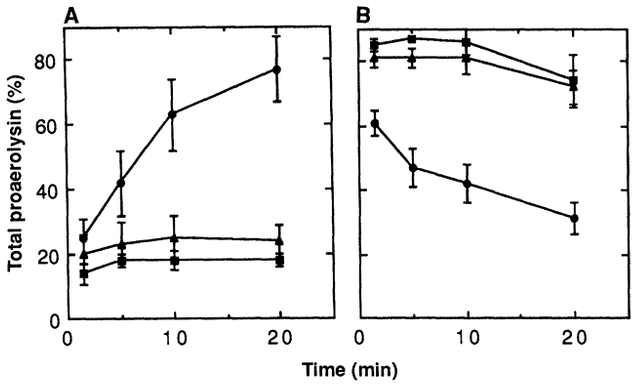

Fig. 2. Time dependence of proaerolysin release-effect of *p*H 5.5 media and CCCP. Cells were grown at 27°C to an OD_{600} of 2.4 to 2.8 in LB medium (*p*H 7.5). They were divided into three equal portions, centrifuged, and resuspended in (●) LB–20 m*M* 1,4-piperazinediethanesulfonic acid (Pipes, *p*H 7.0), (▲) LB–20 m*M* acetate (*p*H 5.5), or (■) LB–20 m*M* Pipes (*p*H 7.0) containing 60 μ*M* CCCP. Chloramphenicol was added (100 μg/ml) to prevent synthesis of new protein. Samples were centrifuged at the time intervals indicated and the cells were shocked (*17*). Proaerolysin was measured in the culture supernatants and in the shockates (*18*). Results are presented as the percentage of total proaerolysin that was measured in these two fractions of the CCCP treated cells at the first time point, 1.5 min after resuspension. Occasionally proaerolysin was also measured in the shocked cells and in the sucrose solution used to condition the cells for shocking. These fractions always contained less than 10% of the total proaerolysin. Results are the means (± SEM) of five experiments. (**A**) Proaerolysin released into the media. (**B**) Proaerolysin remaining in the periplasm.

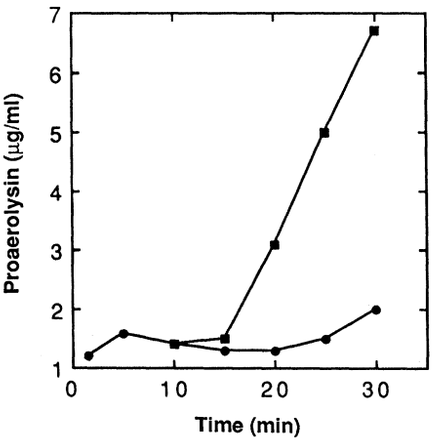

Fig. 4. Release of proaerolysin from AS440 after transfer from *p*H 5.5 media back to *p*H 7.0. Cells were grown as described, pelleted, and resuspended in LB buffered to *p*H 5.5 with acetate as before. The suspension was incubated at 27°C and samples were taken at the time points shown. After 10 min, one-half of the remaining suspension was centrifuged and the cells were resuspended in *p*H 7.0 LB. Release of proaerolysin was followed for an additional 20 min. This is one of three similar experiments; (●) *p*H 5.5 throughout and (■) switched to *p*H 7.0.

the medium would inhibit export. The results in Fig. 2 show that this is exactly what happens. Little or no proaerolysin was released into LB medium buffered at *p*H 5.5 and, as with CCCP, the amount of protoxin in the shockable fraction was not significantly reduced. The amount of proaerolysin released by the cells declined as the *p*H of the medium was reduced below 7.5 (Fig. 3), as would be expected if a gradient of protons were the driving force for export. The inhibition of release shown in Figs. 2 and 3 was not due to an irreversible *p*H-induced change in some property of the cells, because export could be restored by simply transferring the bacteria back to medium at *p*H 7.0 (Fig. 4).

The results obtained with CCCP show that an electrochemical gradient is needed for export of a protein through the outer membrane, and they raise the question of which membrane the gradient is across. If proaerolysin leaves the cell using a TonB-like system, as suggested above, then inhibition by low *p*H must be unrelated to the effect of CCCP, perhaps the result of some reversible change in the structure of the outer membrane. However, both effects can be explained by arguing that a *p*H gradient across the outer membrane is required for export. Low *p*H and a proton gradient of at least one *p*H unit are needed for the entry of diphtheria toxin into the cytosol of eukaryotic cells (*11*). The requirements for the transport of proaerolysin into the culture supernatant of *A. salmonicida* may be similar. A low *p*H on the periplasmic side of the outer membrane may be critical to allow the protoxin to insert, and a proton gradient may provide the driving force necessary for transit. It is quite possible that a transmembrane potential is also required or that a

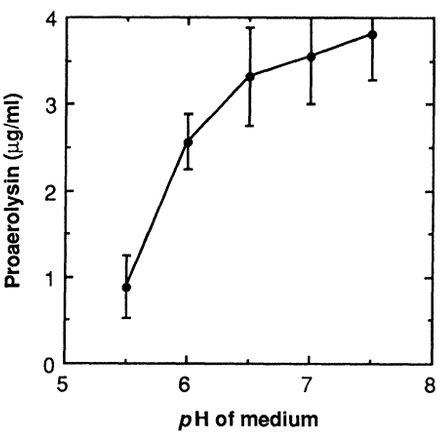

Fig. 3. Dependence of aerolysin release on *p*H. Cells were grown as in Fig. 2. They were pelleted by centrifugation and rapidly resuspended in the same medium buffered to the *p*H values shown [20 m*M* acetate for *p*H 5.5, 20 m*M* 2-[*N*-morpholino]ethanesulfonic acid (MES) for *p*H 6.0, 20 m*M* Pipes for *p*H 6.5 and *p*H 7.0]. Chloramphenicol (100 μg/ml) was present as before. After 10 min incubation at 27°C, the suspensions were centrifuged and proaerolysin was measured in the supernatants (*18*). Results are the means (± SEM) of six experiments. Release is expressed as micrograms per milliliter of cell suspension.

helper protein analogous to the B subunit of diphtheria toxin facilitates transfer through the membrane. The presence of either would explain why transport does not occur from the periplasm back across the inner bacterial membrane.

Like other Gram-negative bacteria, *A. salmonicida* has proteins in its outer membrane that are capable of forming holes in artificial lipid bilayers (*12*). If an outer membrane gradient is required for export, the question arises of how it can be maintained across a membrane filled with hole-forming proteins. The Donnan equilibrium across the outer membrane of *A. salmonicida* has not been characterized; however, regardless of its size, it cannot play a major role by itself, because if this were the case, CCCP would have no effect on export from the periplasm. Perhaps there are zones in the outer membrane that contain no porins, or perhaps the holes formed by the porin proteins are gated, either by a membrane potential associated with the Donnan equilibrium or by means of a regulatory protein analogous to the one recently proposed to modulate the gating of mitochondrial porin (*13*). The inability of *E. coli* to generate a gradient across the outer membrane because it cannot close its porin channels (*14*) may distinguish it from exporting species.

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18. Samples (usually 20 μl) were diluted to 85 μl with phosphate-buffered saline (PBS; 0.85% NaCl, 10 m*M* phosphate, *p*H 7.4) containing 1 μg of trypsin per milliliter. Five microliters of 1*M* tris, *p*H 8, were added to raise the *p*H of the low *p*H samples. After 10 min at room temperature to convert proaerolysin to aerolysin, 90 μl of PBS containing 0.1% bovine serum albumin were added, and the samples were serially diluted 1:2 with the same buffer in microtiter plates with V-shaped bottoms. Each well then received 90 μl of 0.8% (v/v) human erythrocytes, and the plates were incubated for 60 min at 37°C. After incubation, the plates were centrifuged and 135-μl samples of the supernatants were removed from wells in which partial hemolysis had occurred. They were diluted with 850 μl of PBS and their absorbances were read at 413 nm. The amount of aerolysin in each sample was determined from a standard curve prepared in the same microtiter plate with known amounts of purified aerolysin. Neither CCCP nor any of the buffers interfered with the assay system used to measure proaerolysin.
19. We thank S. P. Howard, S. MacIntyre, and F. Pattus for useful criticism and stimulating discussion and M. Cahill for help in preparation of the manuscript. Supported by a grant from the National Sciences and Engineering Research Council of Canada.

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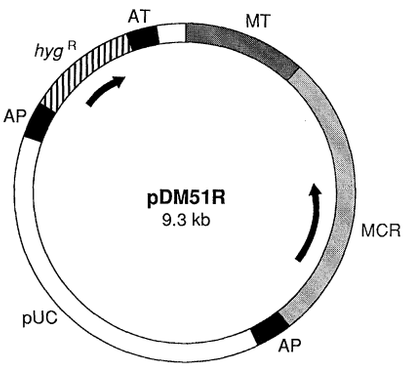

Fig. 1. Physical map of the myosin head fragment expression vector pDM51R. The expression vector is a derivative of the hygromycin resistance vector pDE102 (*6*) carrying 2.6 kb of 5′ coding region of myosin (MCR). The MHF expression cassette lies in the opposite orientation (shown by arrows) to the hygromycin phosphotransferase gene (hyg^R), and the two genes are separated in this construct by 1.1 kb of *mhc*A terminator sequence (MT) and 0.3 kb of *act*-15 terminator sequence (AT). Both the MCR and hyg^R sequences are controlled by the *act*-15 promoter (AP). The white segments represent pUC119 sequences (pUC).

Expression and Characterization of a Functional Myosin Head Fragment in *Dictyostelium discoideum*

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The isolated head fragment of myosin is a motor protein that is able to use energy liberated from the hydrolysis of adenosine triphosphate to cause sliding movement of actin filaments. Expression of a myosin fragment nearly equivalent to the amino-terminal globular head domain, generally referred to as subfragment 1, has been achieved by transforming the eukaryotic organism *Dictyostelium discoideum* with a plasmid that carries a 2.6-kilobase fragment of the cloned *Dictyostelium* myosin heavy chain gene under the control of the *Dictyostelium* actin-15 promoter. The recombinant fragment of the myosin heavy chain was purified 2400-fold from one of the resulting cell lines and was found to be functional by the following criteria: the myosin head fragment copurified with the essential and regulatory myosin light chains, decorated actin filaments, and displayed actin-activated adenosine triphosphatase activity. In addition, motility assays in vitro showed that the recombinant myosin fragment is capable of supporting sliding movement of actin filaments.

MYOSINS CONSTITUTE A FAMILY of diverse proteins that bind to actin and have Mg^{2+}-dependent adenosine triphosphatase (ATPase) activity that is stimulated by actin at low ionic strength. Interaction of myosins with actin in the presence of adenosine triphosphate (ATP) results in the conversion of chemical energy into mechanical force and displacement. Myosins occur in nearly every eukaryotic cell examined, where they participate in many fundamental cellular processes ranging from muscle contraction to cytokinesis. Two subgroups of myosins are known. The first group consists of the double-headed or conventional myosins, genereally referred to as myosin or sometimes as myosin II. The second group consists of the single-headed or unconventional myosins, referred to as myosin I. In this report we will only refer to conventional myosins, all of which show the same structural pattern. They consist of two heavy chains (~ 200 kD each) and two pairs of light chains (15 to 20 kD each). The NH_2-terminal half of the myosin heavy chain (MHC) forms the globular head, which contains the binding sites for the myosin light chains, and the COOH-terminal half forms the extended coiled-coil rod. The globular head fragment of the myosin molecule, also called subfragment-1 or S1, can be released as a soluble fragment by proteolytic cleavage of myosin and has the catalytic and actin-binding properties of the myosin molecule (*1, 2*). It has been shown that S1 alone is sufficient to cause sliding movement of actin filaments in vitro (*3*).

Understanding the mechanism by which myosin catalyzes the transduction of energy stored in chemical bonds into mechanical work will require knowledge of the high-resolution structure of S1 and the ability to manipulate the protein in specific manners at the molecular level. The S1 crystals obtained by Rayment and Winkelmann (*4*) should help in determining the high-resolution structure of the molecule. However, a source of S1 without the inherent heterogeneity of preparations made by proteolytic digestion and that allows the introduction of specific alterations into the molecule by molecular genetic approaches would be desirable. We have addressed this problem by exploring the use of the cellular slime mold *Dictyostelium discoideum* as an expression system for myosin fragments.

The plasmid pDM51R (Fig. 1), which was constructed to achieve expression of a myosin head fragment (MHF) nearly equivalent to S1, carries a translational fusion of the eighth codon of the *Dictyostelium act*-15 gene to the second codon of the *Dictyostelium mhc*A gene. The polypeptide encoded by this fusion has 871 amino acids and extends 46 amino acids beyond the proline residue that marks the region of proteolytic cleavage in muscle myosin. The vector also contains a bacterial hygromycin resistance gene for selection in *Dictyostelium* (*5*) as well as pUC119 sequences (*6*) for selection and autonomous replication in *Escherichia coli*. Transformations of *Dictyostelium* axenic strain AX2 by the calcium phosphate precipitate technique were carried out according to the modified protocol described by Egelhoff *et al.* (*5*). Transformation efficiencies of $\sim 10^{-6}$ were observed. As pDM51R cannot replicate autonomously in *Dictyostelium*, all stable transformants must result from an integration event. The relative amounts of MHF produced by the transformants were estimated from immunoblots of whole-cell

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