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Characterization of energetically functional inverted membrane vesicles from *Corynebacterium glutamicum*

S. Schrempp, N. Bayan, E. Shechter*

Laboratoire des Biomembranes, Université de Paris Sud, 91405 Orsay Cedex, France

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Abstract We show that inverted membrane vesicles from *Corynebacterium glutamicum*, a Gram-positive bacterium, are able to generate and maintain an electrochemical gradient of protons in response to the addition of NADH. This result indicates that the respiratory chain is intact and that the vesicles are reasonably impermeable to protons. These membrane vesicles may be the starting point for in vitro translocation studies of proteins in Gram-positive bacteria.

Key words: Gram-positive bacterium; Membrane vesicle; Membrane potential; Protein translocation

1. Introduction

In vitro translocation systems have been of great use in the elucidation of the molecular mechanism of protein transport across membranes in eucaryotes and procaryotes. In the case of *E. coli*, a Gram-negative bacterium, the system consists of inverted vesicles obtained by physical treatment (high pressure) of spheroplasts. The precursor form of proteins normally translocated, in vivo, across the cyctoplasmic membrane, may be imported, in vitro, into these vesicles. Although only a small number of proteins are efficiently translocated in vitro, the use of such systems has greatly helped in determining the energy requirement for the translocation (ATP and the proton electrochemical potential) [1–3] and the role of the various components involved in the translocation machinery (sec proteins) [4,5].

Our knowledge regarding the secretion of proteins in Gram-positive bacteria is limited. Most studies have been performed with *Bacillus subtilis*. It has been shown that secreted proteins are synthesized in the form of precursors with a signal sequence reminiscent of that of periplasmic proteins in Gram-negative bacteria [6]. Moreover, some Gram-positive bacterial genes, homologous to sec genes in *E. coli*, have been cloned and shown to be able to complement sec mutations in this strain [7–11]. All this suggests that a common mechanism may exist for the secretion of proteins in Gram-positive bacteria and for the translocation of proteins across the cytoplasmic membrane in Gram-negative bacteria. This, however, remains to be proven.

The elucidation of the molecular mechanism of protein secretion in Gram-positive bacteria is hampered by the difficulty in obtaining secretion mutants and by the absence, despite much effort, of an in vitro translocation system. Recently an in vitro processing assay was developed for proteins secreted by *Bacillus subtilis*. Unfortunately this system is ineffective for translocation [12]. The central part of an in vitro system is inverted membrane vesicles derived from the cytoplasmic membrane. The vesicles should be impermeable to protons and be able to generate and maintain an electrochemical gradient of protons (energetically functional vesicles). We have shown recently that *Corynebacterium glutamicum*, a Gram-positive bacterium, secretes large amounts of 2 proteins (PS1 and PS2) [13,14] indicating that it possesses an efficient secretion machinery. We have described some characteristics of the in vivo secretion of PS1 [15]. We report here the characterization of energetically functional inverted vesicles from *Corynebacterium glutamicum*. These may be the starting point for in vitro translocation studies of secreted proteins.

2. Materials and methods

2.1. Preparation of membrane vesicles

Protoplasts were prepared by the method of Yeh et al. [16] as modified by Bonamy (personal communication). Briefly, cells were grown in BHI medium supplemented with 10% succinate, 20 mM $MgSO_4$ and adjusted to pH 8.5. When the culture reached an OD_{570} of 0.5, penicilline G (0.3 U/ml) was added and the incubation was continued for 2 h at 34°C. The cells were centrifuged and resuspended in fresh medium containing 2 mg/ml of lysozyme. The incubation was continued for 20 h at 34°C with slow agitation during which time protoplasts are formed. Their appearance can be monitored by optical microscopy.

The protoplasts were recovered by low speed centrifugation, washed first in BHI medium and then in phosphate buffer (50 mM KPO_4, pH 7.0) containing 0.5 M saccharose, 1 mM PMSF and 1 mM DTT. The protoplasts were resuspended in 1/50 of the original volume in the phosphate buffer and then broken by two runs through a French press at 8000 psi. After low speed centrifugation (removal of unbroken cells and debris), the suspension was centrifuged at 100,000 × *g* for 20 min in a TLA 100.3 rotor. The pellet containing the membrane vesicles was resuspended in 50 mM KPO_4, pH 7.5 supplemented with 5 mM $MgSO_4$. The vesicles were stable for several months at −80°C.

2.2. Determination of $\Delta\Psi$ and ΔpH

The formation of $\Delta\Psi$ in membrane vesicles was monitored by following the fluorescence quenching of oxonol V (λ excitation = 580 nm; λ emission = 620 nm) [17]. The formation of ΔpH was monitored by following the fluorescence quenching of quinacrine (λ excitation = 420 nm; λ emission = 500 nm) [18]. Membrane vesicles (100 to 200 μg protein/ml) were mixed with oxonol (3 μM final concentration) or quinacrine (3 μM final concentration) in a phosphate buffer (50 mM KPO_4, 5 mM $MgSO_4$ pH 7.5). NADH (1 mM final concentration) was added directly into the fluorescent cuvette and changes in fluorescence were recorded as a function of time.

Flow dialysis was performed as described by Reenstra et al. [19]. Membrane vesicles were suspended in the dialysis buffer (50 mM KPO_4

*Corresponding author. Fax: (33) 69 85 37 15.

Abbreviations: BHI, brain heart infusion; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; DCCD, dicyclo-hexylcarbo-diimide; PMS, phenazine methosulfate.

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pH 7.5, 5 mM $MgSO_4$) at 7.5 mg/ml. One ml of this suspension was placed in the upper chamber of the flow dialysis apparatus. The lower chamber was perfused with the dialysis buffer. Both chambers were continuously oxygenated. [^{14}C]Thiocyanate (10 μM final concentration, 2 GBq/mmol) was added to the upper chamber. After equilibration of the labeled solute, NADH (1 mM final concentration) was added. Fractions of 1.6 ml were collected from the lower chamber and the radioactivity determined by liquid scintillation counter. $\Delta\Psi$ was calculated assuming, as for *E. coli*, an internal volume of 1.1 μl/mg of membrane protein [19].

3. Results

The cytoplasmic membrane of *Corynebacterium glutamicum* possesses a respiratory chain which is able to generate an electrochemical gradient of protons when supplemented with an adequate electron donor. The gradient consists of an electrical component (negative inside the cell) and a chemical component (basic inside the cell). In intact cells, and at pH 7.0, these are respectively −140 mV and 0.5 pH unit [15]. Upon addition of an electron donor to inverted membrane vesicles, the chain, if functional, will generate an electrochemical gradient of protons ($\Delta\Psi$ positive inside the vesicle; ΔpH acidic inside the vesicle). The magnitude of these gradients depends upon the integrity of the respiratory chain and upon the impermeability of the membrane to protons, i.e. the integrity of the inverted vesicles.

An electron microscopy image of freeze-fractured membrane vesicles is presented in Fig. 1. It displays a population of vesicles with a mean diameter of 80 nm. Addition of NADH to an aerated suspension of membrane vesicles results in oxygen consumption (Fig. 2). The oxygen concentration, determined using a Clark electrode, decreases as a function of time until the suspension becomes anaerobic. Consumption of oxygen is inhibited by addition of cyanide. This result indicates that an electron transfer takes place between NADH and oxygen through the respiratory chain. Succinate is oxidized more slowly while ascorbate/PMS, an artificial electron donor, is oxidized more rapidly than NADH (data not shown). With NADH as the electron donor, the oxygen consumption was between 200 and 300 nmol of O_2 per minute per mg of membrane protein depending on the preparation. This value compares well with those reported for inverted membrane vesicles of *E. coli* [20].

The generation of $\Delta\Psi$ and ΔpH can be followed by measuring the fluorescence extinction of fluorescent probes as they accumulate inside the vesicle. In this study, we used the lipophilic anion oxonol and the weak base quinacrine to detect the generation of $\Delta\Psi$ (positive inside) and ΔpH (acidic inside), respectively.

Membrane vesicles were incubated with oxonol or quinacrine at room temperature directly in the fluorescent cuvette. Fluorescence was monitored as a function of time. Addition of NADH to these suspensions resulted in the immediate decrease of the quinacrine (Fig. 3A) or oxonol (Fig. 3B) fluorescence. This quenching was abolished by the addition of cyanide (data not shown), CCCP (Fig. 3B), or when the solution became anaerobic (Fig. 3A,B). We conclude that the fluorescence quenching of oxonol and quinacrine indicates the formation of a $\Delta\Psi$ (positive inside the vesicles) and a ΔpH (acidic inside the vesicles), respectively. $\Delta\Psi$ is generated more rapidly than ΔpH; the slow decrease of $\Delta\Psi$ after its rapid formation is the result of its conversion into the more slowly generated ΔpH.

Cyanine is a cationic fluorescent probe which will accumulate inside vesicles upon the generation of a $\Delta\Psi$, negative inside [21]. We did not observe a quenching of fluorescence using

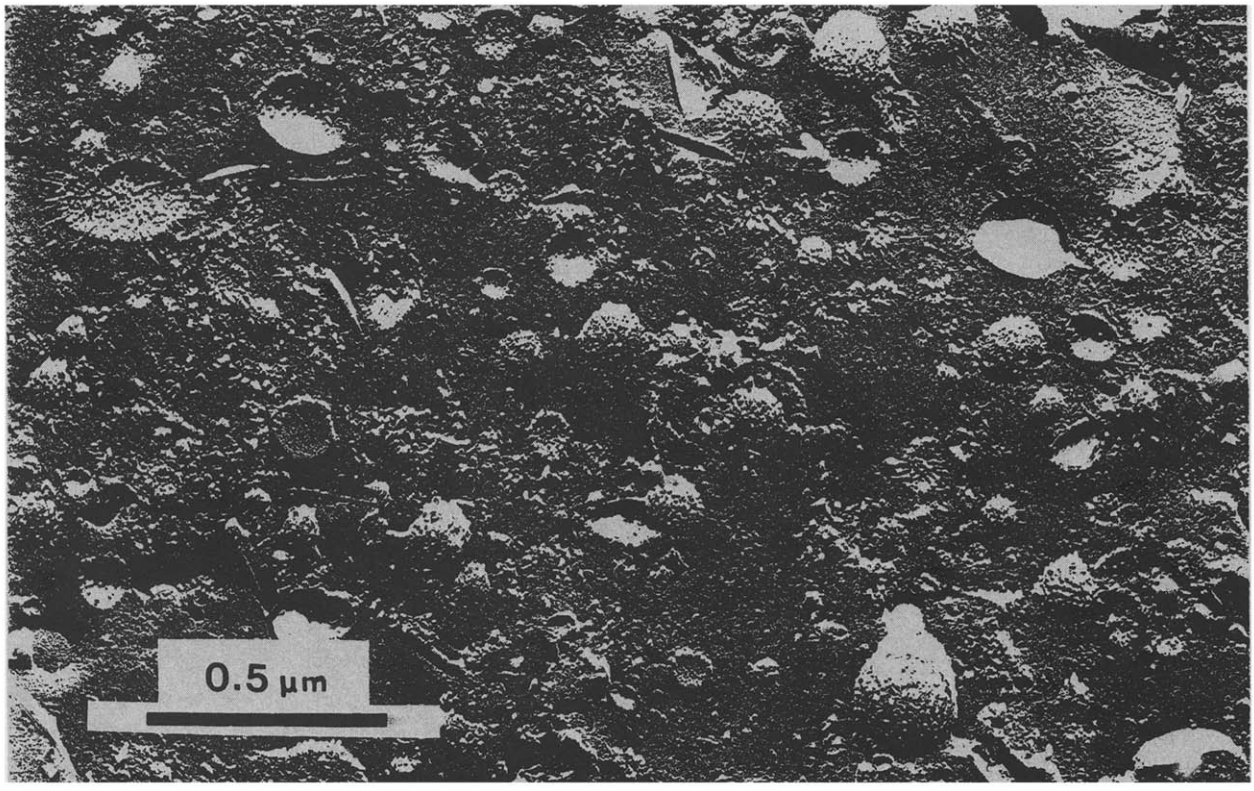

Fig. 1. Electron microscopy image of freeze-fractured membrane vesicles of *Corynebacterium glutamicum*.

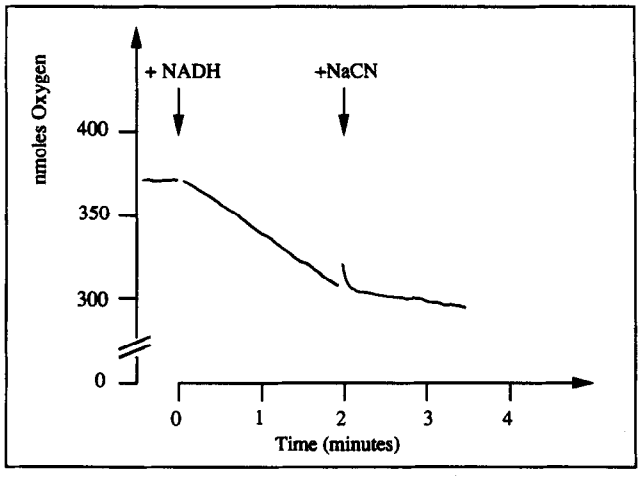

Fig. 2. Oxygen consumption of membrane vesicles. Rates of oxygen uptake were determined with the Clark electrode of a YSI model 53 oxygen monitor. The reaction was initiated by the addition of NADH and inhibited by NaCN. Membrane vesicles were used at a concentration of 130 μg protein/ml in a total volume of 1.5 ml.

cyanide as a probe (data not shown). Thus, the membrane preparation consists predominantly of inverted vesicles.

In inverted membrane vesicles of *E. coli*, an electrochemical gradient of protons may also be obtained by addition of ATP. Under these conditions, the H^+ ATPsynthase will run in the direction of ATP hydrolysis coupled with an inward movement of protons. We were not able to generate an electrochemical gradient of protons by the addition of ATP to inverted vesicles of *Corynebacterium glutamicum* (data not shown) indicating that the ATPsynthase/ATPase is impaired. An impaired H^+ ATPsynthase could be the result of an alteration in the F1 moiety of the complex which renders the membrane permeable to protons through its F0 moiety, thus opposing the formation of $\Delta\Psi$ and ΔpH. If this were true, addition of DCCD, an inhibitor of F0 which blocks all proton movement across F0, should allow the formation of larger $\Delta\Psi$ and ΔpH. Indeed, DCCD treated membranes exhibited higher values of $\Delta\Psi$ (Fig. 4) and ΔpH (data not shown) than those obtained with untreated membranes. Interestingly, DCCD treated membranes remained aerobic longer than untreated ones (Fig. 4). This resulted from a slower rate of respiration in the presence of DCCD (inset Fig. 4). This was not the consequence of respiratory control (CCCP had no effect on the rate of respiration (inset Fig. 4)) but probably reflected an interaction of DCCD with some components of the respiratory chain.

Fluorescence quenching of oxonol and quinacrine are indirect and qualitative methods used to follow the formation of $\Delta\Psi$ and ΔpH. Quantitative information may be attained by the direct determination of the accumulation of a weak hydrophilic anion (thiocyanate) or a weak hydrophilic base (methylamine). We used flow dialysis to follow the accumulation of [^{14}C]thiocyanate in *Corynebacterium glutamicum* inverted vesicles treated with DCCD (Fig. 5, trace A). For comparative purposes, we present data obtained with *E. coli* inverted vesicles (Fig. 5, trace B). The experimental conditions for flow dialysis are very different from those used for the fluorescence experiments. In particular, flow dialysis requires high concentrations of membrane vesicles and thus, the membrane suspension becomes very rapidly anaerobic, even if previously oxygenated. Nonetheless, we were able to detect the formation of $\Delta\Psi$. We calculated a minimum $\Delta\Psi$ of 80 mV for *Corynebacterium glutamicum*

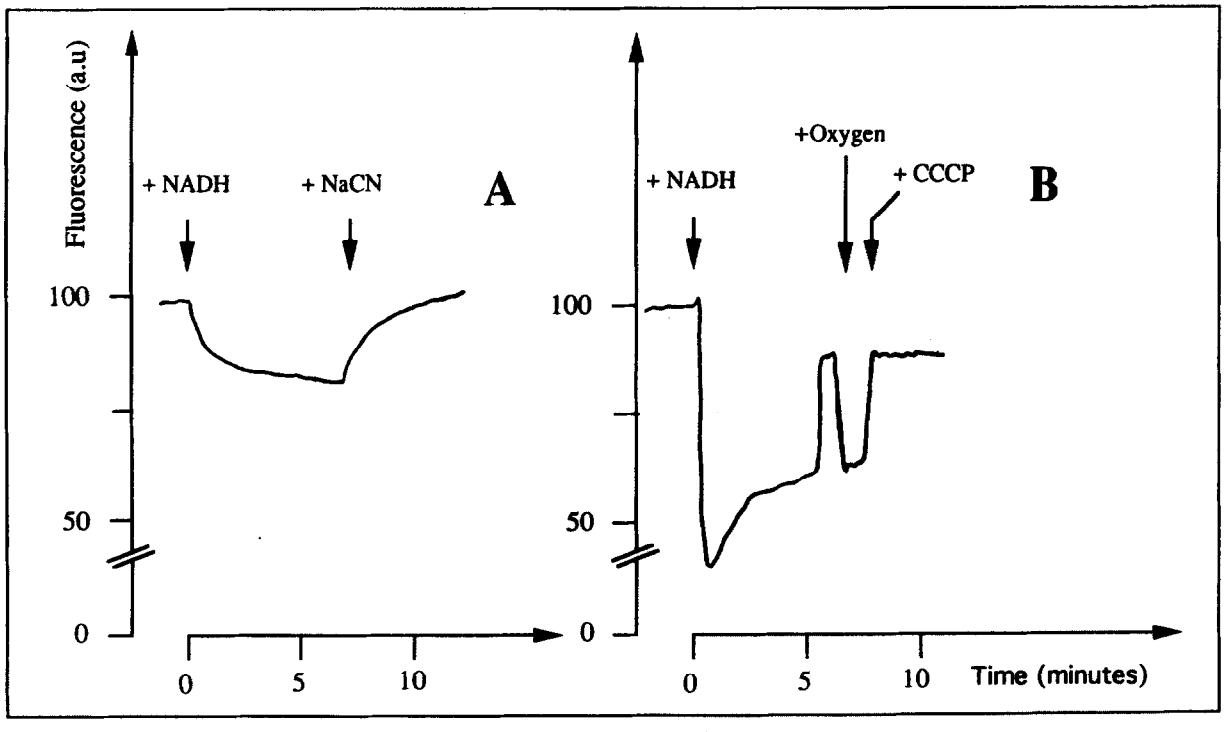

Fig. 3. Formation of ΔpH and $\Delta\Psi$. The formation of ΔpH and $\Delta\Psi$ was followed by monitoring the fluorescence quenching of quinacrine (panel A) and oxonol V (panel B), respectively. ΔpH and $\Delta\Psi$ were maintained until oxygen was depleted or until NaCN or CCCP were added. Membrane vesicles were used at a concentration of 200 μg protein/ml in a total volume of 1 ml.

membrane vesicles (assuming, as for *E. coli*, an internal volume of 1.1 ml per mg membrane protein [19]). This value is smaller than that generated by inverted vesicles of *E. coli* ($\Delta\Psi$ of 110 mV). We were not able to detect the formation of ΔpH. This is probably due to the fact that it was generated more slowly than $\Delta\Psi$ and that the suspension became anaerobic before its formation.

4. Discussion

Energetically functional inverted vesicles derived from the cytoplasmic membrane of *E. coli* are relatively easy to obtain. The preparation requires the passage through a French press, either of whole cells, or of spheroplasts obtained by a mild treatment of the cells with lysozyme. In the case of *Corynebacterium glutamicum*, treatment of cells by the French press did not give efficient breakage of the cells as monitored by measuring the optical density of the suspension. Treatment with lysozyme did not result in the formation of protoplasts as judged by optical microscopy. The lysozyme treatment did perturb the cell wall and a subsequent passage through a French press resulted in the formation of membrane vesicles. These, however, were not energetically functional and we could not generate and maintain an electrochemical gradient of protons (data not shown). We found that the preparation of energetically functional membrane vesicles absolutely required formation of protoplasts which could be obtained only after the treatment which we described in section 2.

This harsh treatment may be responsible for the non-functionality of the F_0F_1 ATPase and the smaller $\Delta\mu_H$ generated in inverted membrane vesicles from *Corynebacterium glutamicum* as compared to that generated in inverted vesicles from *E. coli*.

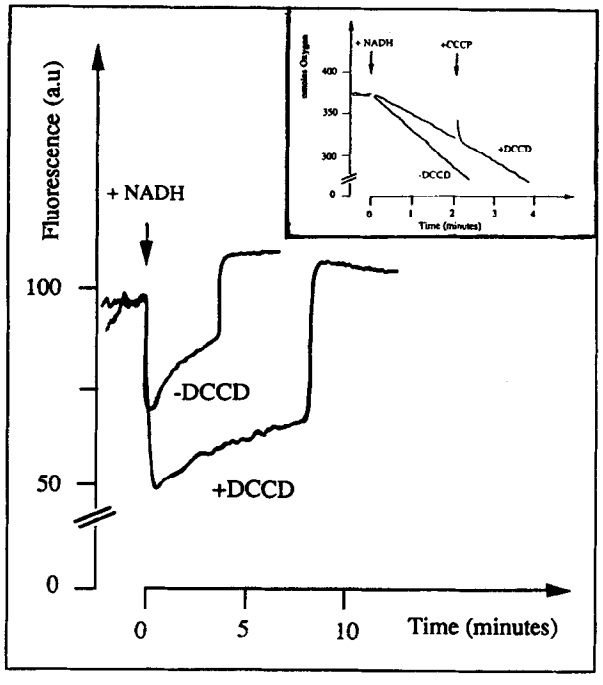

Fig. 4. Influence of DCCD on $\Delta\Psi$. $\Delta\Psi$ was determined as in Fig. 3, for untreated membrane vesicles and membrane vesicles treated with DCCD. Membrane concentration was 100 μg protein/ml in a total volume of 1 ml. Inset: in parallel experiments, the rates of oxygen uptake of the two membrane preparations were determined.

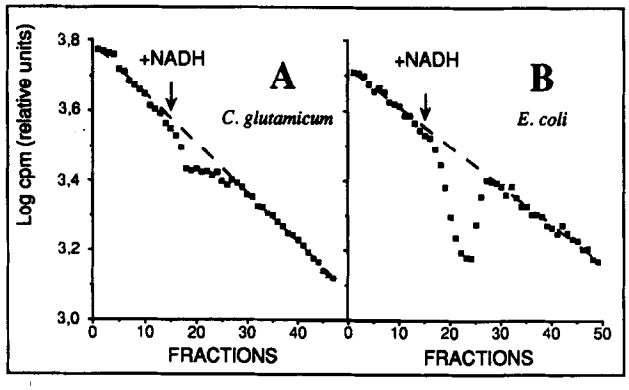

Fig. 5. Determination of $\Delta\Psi$ by flow dialysis. Accumulation of labeled thiocyanate was followed upon the addition of NADH in membrane vesicles derived from *Corynebacterium glutamicum* (panel A) or from *E. coli* (panel B). Membrane concentration was 7.5 mg protein/ml in a total volume of 0.8 ml.

Nevertheless, the membrane preparation can be energized. It should be stressed that translocation can still take place in membrane vesicles of *E. coli* artificially stripped from the F1 part of the ATPsynthase, provided the F0 part is blocked by DCCD [22].

In vitro translocation systems are complex. They require, in addition to energetically functional vesicles, a cell free transcription and/or translation system of a protein whose structure is competent for in vitro translocation and various soluble factors, none of which have been characterized in the case of Gram-positive bacteria. The membrane vesicle preparation which we describe here should prove useful in the elaboration of a complete in vitro translocation system for Gram-positive bacteria.

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