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Immunoelectron microscopic localization of the F_1F_0 ATPase (ATP synthase) on the cytoplasmic membrane of alkalophilic *Bacillus firmus* RAB

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Evidence that the F_1F_0 ATPase (ATP synthase) of alkalophilic *Bacillus firmus* RAB is localized exclusively on the cytoplasmic membrane was obtained by immunogold electron microscopy using a highly specific polyclonal antibody against the β subunit of *Escherichia coli* F_1F_0 ATPase. The energetic problem faced by cells of *B. firmus* RAB growing oxidatively at pH 10.5 despite a low protonmotive force across the cytoplasmic membrane cannot, therefore, be circumvented by localization of energy transducing functions on hypothetical internal membranes.

It is well established that cells of obligately aerobic, extremely alkalophilic bacilli growing at pH 10.5 catalyze normal rates of oxidative phosphorylation, using a proton-translocating F_1F_0 ATPase, despite a low protonmotive force across the cytoplasmic membrane [1–3]. The low protonmotive force results from the maintenance of a cytoplasmic pH that is a full 2 pH units lower than the optimal pH of the growth medium for alkalophiles. We have considered the possibility that such organisms bypass this bioenergetic problem by carrying out energy transducing functions on a hypothetical internal membrane system or organelle whose protonmotive force could be generated independently from the cytoplasmic membrane and which might be comparable to that found in mitochondria or conventional bacteria such as *Escherichia coli*. Although no internal membranes were found in an electron microscopic study of thin sections of alkalophile cells [4], we believed it was important to visualize the subcellular location of alkalophile ATP synthesis directly. This has been achieved by immunogold electron microscopy of ultrathin sections of alkalophile cells using antibodies against the β subunit of the F_1F_0 ATPase of *E. coli*. The bound gold particles were found to be associated with the cytoplasmic membrane. We conclude that ATP synthesis in alkalophiles occurs only on the cytoplasmic membrane and not on any putative internal membranous structure.

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Bacillus firmus RAB was grown at pH 10.5 as described [5], harvested and washed once with phosphate buffer at pH 6.9. The cells were fixed on ice for 1 h with 0.2% glutaraldehyde and 0.3% formaldehyde and washed with phosphate buffer containing 10 mM glycine to block free aldehyde groups. Methanol was used to dehydrate the cells: 15% for 30 min on ice, 30% for 30 min on ice, 50% for 30 min at −25°C, 70% for 30 min at −35°C, 90% for 30 min at −35°C, and 100% for 30 min at −35°C. This was followed by 1 part 100% methanol and 1 part Lowicryl K4M resin at −35°C for 1 h, 1 part 100% methanol and 2 parts resin at −35°C for 1 h, and resin alone at −35°C overnight. The samples were put into gelatin capsules, filled with fresh resin and polymerized with UV light for 24 h at −40°C and 24 h at room temperature. Ultrathin sections were cut and mounted on formvar covered grids and incubated with rabbit-anti-β-antibody either at 4°C overnight or at room temperature for 2 h. After rinsing with buffer, the sections were incubated for 1 h with either goat-anti-rabbit-IgG-gold complex or protein A-gold complex, rinsed again with buffer and distilled water and air dried. Before poststaining, the sections were put on drops of 0.1 M EDTA for 2 min, blotted on filterpaper and air dried. Post-staining was done with 4% uranyl acetate (pH 4.5) for 2 min.

The micrographs shown in Fig. 1 (A, C, D, E) demonstrate that extensive labeling of the cytoplasmic membrane occurred when thin sections of *B. firmus* RAB cells were incubated with anti-β-antibody followed by incubation with the appropriate gold complex. Labeling of the membrane took place when either goat anti-rabbit-IgG-gold complex (Fig. 1E) or protein A-

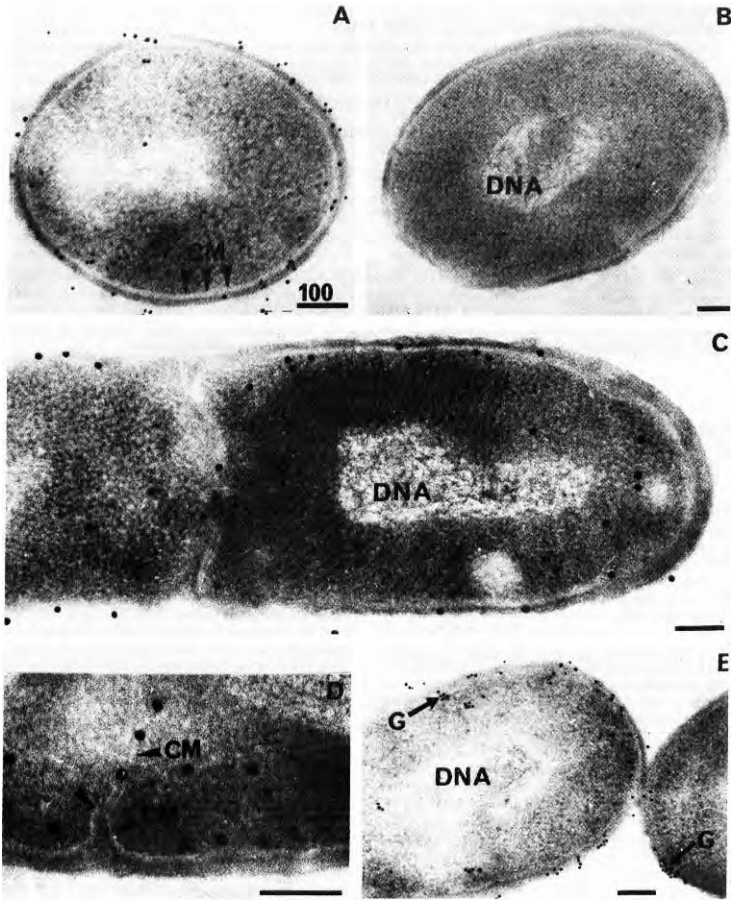

Fig. 1. Demonstration of the localization of the F_1F_0 ATPase of *B. firmus* RAB by immunoelectron microscopy. In panels A, C, D, E, thin sections were treated with anti-β-antibody to *E. coli* F_1 and visualized by incubation with either goat-anti-rabbit-IgG-gold complexes (B, E) or with protein A-gold complexes (A, C, D). The size of the gold complexes were 5 nm (A, B, E) or 15 nm (C, D). A control is illustrated in panel B, in which the specific anti-β-antibody was replaced with nonspecific (normal) goat serum and was visualized using goat-anti-rabbit-IgG-gold complex. Bars represent 100 nm. CM, cytoplasmic membrane; G, gold particle.

gold complex (Fig. 1A, C, D) were reacted with the β-anti-β-antibody complex. It was found that the number of gold particles was significantly greater when 5 nm gold complexes (A, E) were used rather than 15 nm gold particles (C, D), as has been found elsewhere [6,7]. The subcellular distribution of the gold particles was independent of the size or the type of gold complex. A statistical analysis of 100 cells indicated that 80.5% ± 3.5% of the particles were associated with the cytoplasmic membrane and 19.5% ± 3.5% were found in the cytoplasm. Since the cytoplasmic membrane occupies a very small area relative to the area of the cytoplasm, this distribution is good evidence that the F_1F_0 ATPase is located on the cytoplasmic membrane.

The few gold particles observed in the cytoplasm, as shown in Fig. 1A, C, appeared to be randomly distributed and could be accounted for by the presence of newly synthesized β subunit or degraded β subunit. We have shown that the anti-β-antibody reacted only with one subunit of the appropriate size in the purified F_1 ATPases of *B. firmus* RAB and *Bacillus subtilis* BD99 [8,9]. We have also noted a highly specific reaction of this antibody in protein immunoblots of whole cell extracts of *B. subtilis* and *Vibrio alginolyticus* probed with the anti-β-antibody, yielding a single reactive band of about 50000 molecular weight (Hicks, D.B., unpublished results). Control experiments demonstrated the specificity of the immunogold labeling. When thin sections were incubated with normal goat serum followed by goat-anti-rabbit-IgG-gold complex, virtually no labeling was observed (Fig. 1B). A second control in which the first antibody was left out resulted in very few gold particles when thin sections were incubated with either goat-anti-rabbit-IgG-gold complex or protein A-gold complex (data not shown).

Clustering of gold particles labeling the membrane was observed when the antibody-gold complex was used (Fig. 1E), but was much less evident when protein A-gold replaced the antibody-gold complex (Fig. 1A, C, D). Clustering with the antibody-gold may have been due to the fact that more than one antibody-gold complex can bind to the specific antibody, whereas only one protein A-gold complex can bind to the Fc-part of the specific antibody. We noted, however, that even when protein A-gold was used, the labeling was not uniform throughout the entire membrane surface. Evidence of clustering has been discussed in several other immunoelectron microscopic studies of integral membrane proteins and has been interpreted in some cases as an artifact [10] and in others as a reflection of the in vivo distribution of the protein in the membrane [11,12]. Artificial clustering may result from migration of the antigen when thin sections are cut from frozen cells after mild fixation [10]. In this work, in which the labeling was carried out subsequent to fixation and embedding, the antigen presumably was not free to migrate. It will be of interest to ascertain whether clustering resulted from other aspects of the procedure or whether there might be some nonrandom localization of the ATPase.

The results shown in Fig. 1 indicate that the F_1F_0 ATPase of alkalophilic *B. firmus* RAB resides exclusively on the cytoplasmic membrane and not on any internal membranous structure. Another possible solution to the energetic problem of alkalophiles is that ATP synthesis is driven by sodium ion electrochemical gradients instead of proton electrochemical gradients. There is, however, a considerable body of work which indicates that in alkalophiles protons and not sodium ions are utilized by the ATP synthase [1–3], and we have recently been able to demonstrate the proton translocating nature of the isolated ATPase reconstituted in proteoliposomes (Hicks, D.B., et al., unpublished results). Thus, our finding that the F_1F_0 ATPase is localized exclusively to the cytoplasmic membrane is consistent with and serves to reinforce the apparent bioenergetic dilemma that alkalophilic bacteria present.

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