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The Abundance of *atp* Gene Transcript and of the Membrane F_1F_0-ATPase as a Function of the Growth pH of Alkaliphilic *Bacillus firmus* OF4

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Molecular biological and biochemical studies of the F_1F_0-ATP synthase of alkaliphilic *Bacillus firmus* OF4 show that the enzyme used at pH 7.5 and pH 10.5 is a unique product of the *atp* operon, expressed at the same levels and yielding an enzyme with the same subunit properties and *c*-subunit/holoenzyme stoichiometry.

The bioenergetics of oxidative phosphorylation in extremely alkaliphilic *Bacillus* species is difficult to describe according to a strictly chemiosmotic model. The maintenance of a cytoplasmic pH below pH 8.5 during growth at pH values of 10.5 and higher reduces the net electrochemical proton gradient (Δp) that is available to energize proton-coupled bioenergetic work. The surprising observation is that the small Δp at pH 10.5, for example, supports similar or higher phosphorylation potentials (ΔGp values) in alkaliphilic *Bacillus firmus* OF4 as observed with cells growing at pH 7.5, although the total Δp is almost three times higher in pH 7.5-grown cells (7, 19). The discordance is even greater when the external pH is elevated above 11 (19). Since neither the use of Na^+ as a coupling ion nor the sequestration of oxidative phosphorylation in organelles accounts for these observations with extreme alkaliphiles, the possibility of a highly variable H^+/ATP coupling stoichiometry has been considered as a way of fitting the data to a chemiosmotic model (13). In a completely chemiosmotic coupling mode, the $\Delta Gp/\Delta p$ ratio should be equal to the H^+/ATP ratio so that a higher H^+/ATP ratio would compensate for a lower Δp. In order to account for the bioenergetic parameters measured in a rigorously controlled chemostat study, the H^+/ATP ratio would have to vary from just above 3 to 13 over the organism's pH range for rapid growth (19). Such a variability in stoichiometry is difficult to envision if a single species of synthase operates over the entire range of growth pH. Prokaryotic ATP synthases, including those of the *Bacillus* species studied to date, are F-type ATPases encoded by operons in which the first cistron is generally a gene (*atpI*) of unknown function, which is followed by the three genes (*atpBEF*) encoding the subunits of the integral membrane F_0 part of the complex and then the five genes (*atpHAGDC*) encoding the catalytic F_1 part (3, 6, 10). Earlier PCR studies failed to indicate more than one *atp* operon or multiple *atpE* genes encoding the *c*-subunit, even though the degenerate primers used were able to amplify the alkaliphile genes, which have specific sequence motifs (10, 11), as well as conventional *atp* genes from unrelated *Bacillus* species (11). However, previous studies did not include a characterization of the actual enzyme found at different pH values or whether there was a pH-dependent change in the level of the synthase, in its subunit structure, or in the *c*-subunit/holoenzyme ratio. In *Enterococcus faecalis*, an increase in the amount of the ATPase was found to be an adaptation to a low external pH (12), making it of interest to examine the possibility of up-regulation by the alkaliphile at high external pH values for growth. No evidence has been presented for a discrete change in *c*-subunit/holoenzyme stoichiometry in connection with physiological or environmental challenges. However, the presence of approximately 9 to 12 copies of this subunit per F-ATPase assembly and the importance of this subunit in the pathway for the coupling ion have led to proposals directly relating a synthetic function and the H^+/ATP stoichiometry to the *c*-subunit/holoenzyme stoichiometry of different classes of ATPases (4).

In this study, total RNA (14) from *B. firmus* OF4 cultures grown at pH 7.5 and pH 10.5 was analyzed by Northern (RNA) blots, using methods described previously (15). A probe consisting of the entire *c*-subunit gene and upstream region hybridized to the full-length (7-kb) transcript of the *atp* operon, giving signals of approximately equal intensity with RNA samples obtained from cells grown at the two pH values (Fig. 1). If anything, in the particular set shown, there was slightly more hybridizing RNA in cells grown at the lower pH. No other bands were detectable, suggesting that transcription from an internal promoter upstream of the *atpE* gene does not occur at either growth pH. Identical results were obtained with a probe containing an internal region of the *a*-subunit gene (data not shown). Primer extension analysis was used to determine the transcriptional start site of the *atp* operon. A single extended product of the same size was obtained with samples of RNA isolated from either pH 7.5- or pH 10.5-grown cells, and the intensities of bands were approximately the same at both pH values, consistent with the results of the Northern blot analyses (data not shown).

Membranes from *B. firmus* OF4 grown at either pH 7.5 or pH 10.5 were isolated, fractionated on denaturing gels, and probed by Western (immunoblot) analysis with antibody against the β-subunit of *Escherichia coli*, using procedures comparable to those described earlier (9). As shown in Fig. 2, there was no difference in the amount of this major F_1-ATPase subunit in membranes from cells grown at the two pH values. The synthase represented 2.2% of membrane protein, being present at 44 pmol/mg of membrane protein in sets of preparations from cells grown at the two pH values. The F_1F_0-ATPase was purified from membranes of pH 7.5- and pH 10.5-grown cells; although not shown, the initial membranes

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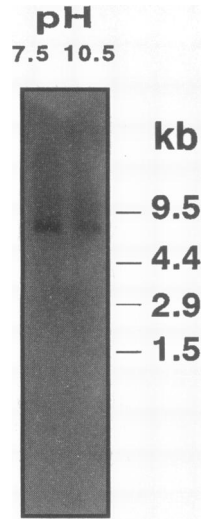

FIG. 1. Northern blot analysis of the *atp* operon of *B. firmus* OF4. Five-microgram samples of total RNA from cells of *B. firmus* OF4 grown at pH 7.5 and pH 10.5 were denatured, electrophoresed, transferred to nylon membranes, and probed with a radiolabeled fragment containing the *atpE* gene. The probe consisted of a 499-bp *Nhe*I-*Ssp*I fragment of pRC3. pRC3 was isolated from a *B. firmus* OF4 *Eco*RI-*Cla*I genomic DNA library constructed in pSPT19 (Boehringer Mannheim) and contains the genes encoding the subunits *i*, *a*, *c*, *b*, and δ of the ATP synthase downstream of the T7 RNA polymerase promoter. Standard molecular biological procedures used in this and other manipulations were conducted as described previously (1, 16). Positions and sizes of RNA standards (Gibco/BRL) are shown on the right.

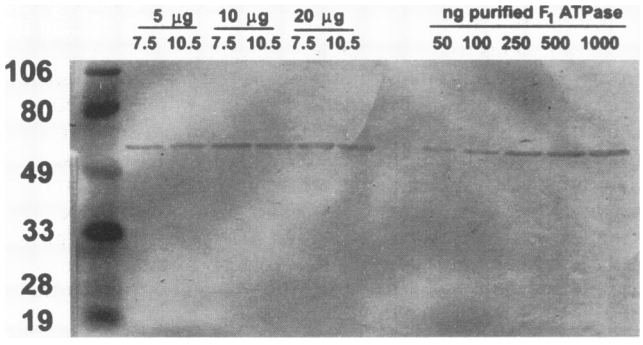

FIG. 2. Quantitative immunoblot of β-subunit (F_1F_0-ATPase) content in membrane vesicles of *B. firmus* OF4. Everted membrane vesicles were prepared essentially as described previously (8). Five-, ten-, and twenty-microgram samples of total membrane protein from *B. firmus* OF4 grown at pH 7.5 and pH 10.5 were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monospecific polyclonal antibody against the β-subunit of *E. coli* F_1. After densitometric analysis of the bands formed from the indicated concentrations of purified *B. firmus* OF4 F_1 (the right half of the blot), a standard curve, from which the concentration of enzyme in membrane samples was calculated, was prepared. Molecular weight standards (in thousands) are on the left.

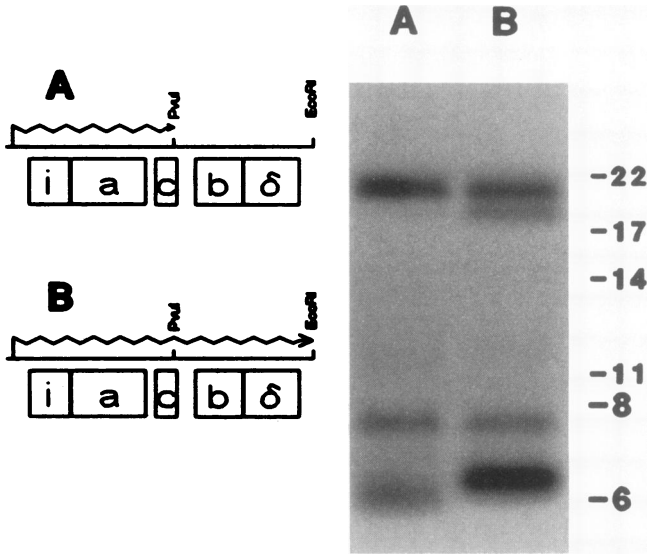

FIG. 3. In vitro transcription and translation of the genes of the *B. firmus* OF4 *atp* operon encoding the F_0 subunits and δ. *Pvu*I (A) and *Eco*RI (B) digests of pRC3 were used for in vitro transcription and translation, which were carried out by using the protocols provided by Promega. Following transcription with T7 RNA polymerase, plasmid DNA was digested with RQ1 DNase and the products of transcription reactions were added directly to an *E. coli* S30 translation system (Promega) containing [^{35}S]methionine. Translation products were separated by SDS-PAGE and visualized by autoradiography. Molecular masses (in kilodaltons) of marker proteins are given on the right.

had comparable ATPase activities and the pattern observed upon gel electrophoresis of the two purified preparations was identical to that found earlier for enzyme from pH 10.5-grown cells (8). The relative amounts and sizes of the subunits were comparable for the two preparations and were reproducible among independent preparations.

The *c*-subunit was only barely discernible or in some cases not detectable in Coomassie-stained gels. To definitively identify the position of this subunit, as well as that of the *a*-subunit, in the gel system, in vitro transcription and translation of the 5′ portion of the *atp* operon of *B. firmus* OF4 were carried out. A 2.9-kb *Cla*I-*Eco*RI fragment of *B. firmus* OF4 DNA containing the *atpIBEFH* genes was placed behind the T7 RNA polymerase promoter of pSPT19. *Pvu*I digestion of this construct results in such a truncation of the *atpE* gene that the *c*-subunit lacks its final nine amino acid residues and the *b*- and δ-subunits are not made. *Eco*RI digestion of the construct leaves all of the genes intact. As shown in Fig. 3, transcription and translation of these digests, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, allowed unambiguous identification of the products of the *atpIBEH* genes, the subunits *i*, *a*, *c*, and δ, respectively. The smallest product of the *Pvu*I-digested construct (Fig. 3A) was smaller than that of the *Eco*RI-digested construct (Fig. 3B), confirming that this was the *c*-subunit in truncated and untruncated forms, respectively. Not detectable in this experiment was the product of the *atpF* gene, the *b*-subunit. This may reflect the use of GUG as the start codon for this gene; the S30 translation system employed in this experiment may not recognize and translate this region appropriately. Having established the electrophoretic mobility of the authentic *c*-subunit, quantitative comparison of the *c*-subunit content of membranes from pH 7.5- and pH 10.5-grown cells of *B. firmus* OF4 was carried out by analysis of gel fractionation of membranes that had been treated with radioactive *N*,*N*′-dicyclohexylcarbodiimide (DCCD). In Fig. 4, the pattern of membrane proteins from one of several independent sets of

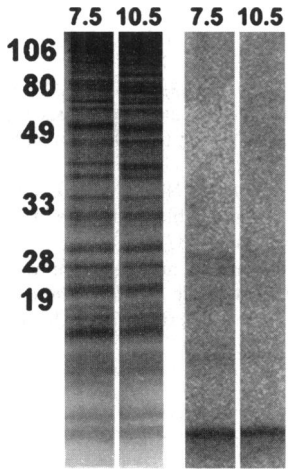

FIG. 4. [^{14}C]DCCD labeling of everted membrane vesicles of *B. firmus* OF4 grown at pH 7.5 and pH 10.5. Everted membrane vesicles were incubated with [^{14}C]DCCD overnight and washed twice as described by others (2, 5). Radiolabeled vesicles were extracted with chloroform-methanol (2:1) and ether precipitated. Ether precipitates were solubilized in SDS sample buffer, loaded on an SDS-PAGE gel (17), and stained for total protein by Coomassie brilliant G (first two lanes), and the gel was dried and exposed to a phosphorimager screen (last two lanes) to detect radiolabeled proteins. The molecular mass of the band corresponding to the *c*-subunit was approximately 7 kDa. Prestained molecular weight standards (in thousands) are indicated on the left.

such membranes is shown side by side with an autoradiogram of the same gel. Although there were some differences in overall membrane protein patterns, some of which are not reproducible in independent sets, the autoradiograms consistently indicated no pH-dependent change in the *c*-subunit content of the membranes. Given the observations that both an F_1 subunit and the *c*-subunit were present in the same amounts in membranes from pH 7.5- and pH 10.5-grown cells, the ratio of *c*-subunit to holoenzyme must also be the same at the two pH values.

Previous studies indicated that the *B. firmus* OF4 *c*-subunit and that of another, unrelated, extremely alkaliphilic *Bacillus* sp. differed from homologs of several nonalkaliphilic bacteria in the regions of the *c*- and *a*-subunits (11, 13). The *c*-subunit was purified by chloroform-methanol extraction (5) of membranes from *B. firmus* OF4 grown at pH 7.5 and pH 10.5 to determine whether partial analysis by microsequencing would indicate that both or only one of the preparations contained the unusual sequence features that had been deduced from the gene sequence. Preparations of the *c*-subunit in chloroform-methanol (2:1) were applied to a polyvinylidene difluoride membrane and subjected to cyanogen bromide cleavage (18); N-terminal amino acid sequencing was performed on a gas-phase sequencer by using standard Edman degradative chemistry. Microsequencing from the N terminus proceeded much more efficiently after treatment of the samples with cyanogen bromide, indicating that a blocked N-terminal methionine is probably present in purified *c*-subunit samples. Subsequent to cyanogen bromide treatment, it was possible to identify 23 residues of sequence from the N terminus and an additional 5 residues that followed the single internal cleavage and appeared as a minor sequence set. Both of these sequences were identical in samples from cells grown at the two pH values; moreover, they conformed precisely to the sequence deduced from the gene sequence. The determined sequence included many residues that are thus far specific to two alkaliphile *c*-subunits. For example, in the first putative transmembrane helix, sequencing identified the following residues (presented as residue and position number): G-5, G-17, A-20, I-21, and A-22. In addition, the unusual second proline (at position 58), near the important carboxylate in the second putative transmembrane helix, was identified in both samples. Thus, the ATP synthase from *B. firmus* OF4 grown at pH 7.5 and pH 10.5 appears to be a unique enzyme that is neither present in unusual or pH-dependent amounts in the membrane nor variable in its ratio or in the species of *c*-subunit. If the alkaliphile uses the enormously variable H^+/ATP stoichiometry required to account for the phosphorylation potentials generated at different values of growth pH, then it does so with a single enzyme. The unlikeliness of this is compounded by the finding that the molar growth yield of *B. firmus* OF4 on limiting malate in continuous culture is actually higher at pH 10.5 than at pH 7.5, the reverse of what would be expected if more protons had to be translocated inward to produce the same amount of ATP (19). Moreover, there are qualitative observations in connection with alkaliphile oxidative phosphorylation that would not be accounted for simply by a variable stoichiometry of coupling (13).

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