The Mother-Cell-Membrane Adenosine Triphosphatase of Sporulating *Clostridium pasteurianum*

David J. CLARKE and J. Gareth MORRIS

Department of Botany and Microbiology, School of Biological Sciences, University College of Wales,
Aberystwyth SY23 3DA, U.K.

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1. Sporulation of *Clostridium pasteurianum* effects several changes in its proton-translocating cell-membrane H^+ -ATPase. Notable among these are the acquisition of susceptibility to activation by trypsin and a changed protein subunit composition. 2. A protein was isolated from the mother-cell membrane that inhibited the ATP phosphohydrolase activity of purified vegetative-cell-membrane H^+ -ATPase [BF_0F_1 complex, which consists of soluble ATPase (BF_1) and the proton-channel component (BF_0)] and rendered it susceptible to trypsin activation. 3. This trypsin-sensitive inhibitor protein had a molecular weight of 10000 and on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was indistinguishable from the novel protein subunit *e* of the mother-cell-membrane ATPase. 4. In bacteriorhodopsin-containing everted membrane vesicles, the specific ATP synthetase activity of the mother-cell-membrane ATPase was significantly greater than that of the vegetative-cell-membrane ATPase. 5. Treatment with trypsin-sensitive inhibitor protein of artificial proteoliposomes containing bacteriorhodopsin and vegetative-cell-membrane H^+ -ATPase (BF_0F_1) significantly increased the specific ATP synthetase activity of this enzyme. 6. The ATP synthetase activity of crude cell-membrane preparations from cultures of *Clostridium pasteurianum* increased during that period in the course of sporulation when the membrane ATP phosphohydrolase was both most rapidly decreasing in specific activity and acquiring its susceptibility to activation by trypsin.

Clostridium pasteurianum is an obligately fermentative anaerobic bacterium whose membrane-associated Mg^{2+} -dependent ATPase (EC 3.6.1.3) may well *in vivo* act unidirectionally as a proton-exporting ATP phosphohydrolase (Morris, 1977). By this means, ATP generated by substrate-level phosphorylation powers the creation and maintenance of the transmembrane protonmotive force, which is exploited to maintain the ionic integrity of the organism and to transport certain fermentable substrates (Riebeling *et al.*, 1975; Booth & Morris, 1975). In a preliminary communication (Clarke & Morris, 1977a) we reported that sporulation in *Cl. pasteurianum* wrought several changes in the composition and properties of its cell-membrane ATPase. The membrane ATP phosphohydrolase of the mother cell differed from that present in vegetatively growing organisms in (i) its lower specific activity, (ii) its susceptibility to activation by brief treatment with trypsin, (iii) its insensitivity to inhibition by the bacteriocin butyricin 7423 and (iv) its changed

polypeptide-subunit composition. Of the changes in the constitution of the enzyme complex, the most obvious was the presence in the purified mother-cell-membrane ATPase [BF_0F_1 complex, which consists of soluble ATPase (BF_1) and the proton-channel component (BF_0)] of a low-molecular-weight ($10000 \pm 10\%$) protein (designated subunit *e*) that was totally absent from the similarly purified vegetative-cell-membrane ATPase complex.

We now report the isolation and purification from the mother-cell membrane of a trypsin-sensitive inhibitor protein that appears to be identical with the novel subunit *e* of the mother-cell-membrane ATPase. We further describe some consequential effects of adding this purified trypsin-sensitive inhibitor protein to a purified preparation of the vegetative-cell-membrane ATPase complex.

Experimental

Organisms

Clostridium pasteurianum 6013 was kindly supplied by Mrs. W. Ego (University of Hawaii, Hono-

Abbreviations used: H^+ -ATPase, H^+ -translocating ATPase; SDS, sodium dodecyl sulphate.

lulu, HI, U.S.A.) as strain W-5, A.T.C.C. 6013. Its granulose-negative mutant strain MR505 has previously been described (Robson *et al.*, 1974).

Growth of cultures

Washed spore suspensions of *Cl. pasteurianum* were prepared as described by Mackey & Morris (1971) and were stored at 2°C. Samples of these spore suspensions were heat-activated before being employed as inocula of suitable volumes of growth medium (Robson *et al.*, 1974). The parent wild-type organism was grown in batch culture in a minimal glucose (2%, w/v) + ammonium salts and vitamins medium (Robson *et al.*, 1974) incubated anaerobically at 37°C under N₂/CO₂ (19:1). Mutant strain MR505 was grown in this glucose minimal medium lacking NH₄⁺ ions but supplemented with casein hydrolysate (1%, w/v). Culture growth was measured by the increase in A₆₈₀, and the initiation and progress of the near-synchronous sporulation was followed by monitoring deposition of granulose and by the production of phase-dark forespores before the formation of phase-bright spores (Mackey & Morris, 1971). In the absence of granulose deposition, the phase-dark forespores of mutant strain MR505 were rendered more easily visible by staining air-dried smears of the culture with Gram's iodine.

Isolation of cell membranes

Vegetative-cell membranes were obtained from organisms harvested in the early exponential phase of culture growth. The cells were anaerobically washed and resuspended in 0.1 M-potassium phosphate buffer, pH 6.4, containing 6 mM-MgSO₄, 15% (w/v) lactose and 0.2 mg of lysozyme (EC 3.2.1.17)/ml. After incubation at 37°C for 40 min, protoplasts were harvested by gentle centrifugation (6000 g for 20 min at 4°C) and were suspended in 90 mM-Tris/H₂SO₄ buffer, pH 7.6, containing 6 mM-MgSO₄, 1 mM-ATP, 20 mM-2-mercaptoethanol and 15% lactose. This suspension was gently ruptured by anaerobic passage at 6 × 10⁷ Pa through a French pressure cell (Aminco, Silver Spring, MD, U.S.A.). Deoxyribonuclease (EC 3.1.4.5) was added to 20 µg/ml and the mixture was incubated at 20°C for 20 min. Cell debris was removed by centrifugation (under N₂) at 5000 g for 15 min at 4°C and then at 16 000 g for 20 min at 4°C. The clarified extract was anaerobically centrifuged at 200 000 g for 20 min at 4°C to yield a pelleted membrane fraction. This was washed and resuspended in the Tris/H₂SO₄ medium, pH 7.6 (described above) but minus lactose.

Mother-cell membranes were similarly obtained from organisms that had accomplished Stage III of sporulation (i.e. engulfment). This stage was normally attained some 2–3 h after initiation of sporu-

lation, when about 80–90% of the organisms contained phase-dark forespores. The protoplast suspension produced by treatment with lysozyme was disrupted by passage at 3.5 × 10⁷–4.8 × 10⁷ Pa through a French pressure cell. After treatment with deoxyribonuclease (as above), the liberated forespores were removed by anaerobic centrifugation at 8000 g for 20 min at 20°C. With the wild-type organism, further low-speed centrifugation (under N₂) was undertaken to remove the massive granulose fraction, which, of course, was not present in extracts of sporulating cells of mutant MR505. Further centrifugation (200 000 g for 20 min at 4°C) of the clarified mother-cell extract yielded a pelleted mother-cell-membrane fraction, which was anaerobically washed and resuspended in the Tris/H₂SO₄ buffer, pH 7.6, containing 6 mM-MgSO₄, 1 mM-ATP and now 1 mM-2-mercaptoethanol.

Isolation and purification of membrane ATPase (BF₀F₁ complex)

Preparations of (a) vegetative-cell-membrane ATPase and (b) mother-cell-membrane ATPase were obtained and purified by the procedure previously described for the vegetative-cell-membrane ATPase (Clarke & Morris, 1976, 1977b; Clarke *et al.*, 1979). Briefly, this involved anaerobic Triton X-100 extraction of washed cell membranes, filtration through a column of Sepharose CL-4B, ion-exchange chromatography on DEAE-Sepharose CL-6B followed by substitution of 0.2% cholate for Triton X-100 during column filtration through Sephadex LH-20 with an eluting mixture of 90 mM-Tris/H₂SO₄ buffer, pH 7.6, containing 6 mM-MgSO₄, 1 mM-ATP plus 20 mM-2-mercaptoethanol (for the vegetative-cell-membrane ATPase) or 1 mM-2-mercaptoethanol (for the mother-cell-membrane ATPase) plus 50% (w/v) ethanediol and 0.2% sodium cholate. Ultrafiltration of those pooled fractions that contained NN'-dicyclohexylcarbodi-imide-sensitive ATP phosphohydrolase activity yielded a concentrated purified enzyme preparation that could be stored under liquid N₂.

Assay of ATP phosphohydrolase activity

ATP phosphohydrolase activity was assayed: (a) by measuring release of P_i from ATP by using a modification of the procedure of Muñoz *et al.* (1968) in which production of phosphate was followed semi-continuously (Clarke & Morris, 1976); (b) by monitoring the proton release that accompanies hydrolysis of ATP, by using essentially the procedure described by Lloyd & Edwards (1976). In each case the unit of ATP phosphohydrolase activity was defined as 1 µmol of product formed/min at 37°C.

Trypsin activation of ATP phosphohydrolase activity

A sample (0.4 ml) of the cell-membrane preparation (about 2 mg of protein) was incubated with 8 μ g of trypsin (11400 Bz-Arg-OEt units/mg) at 37°C. Portions (50 μ l) were removed at intervals and their ATP phosphohydrolase activities were immediately assayed in the presence of an excess of (soya-bean) trypsin-inhibitor protein.

ATP-dependent enhancement of 8-anilino-naphthalene-1-sulphonic acid fluorescence

The ATP-dependent enhancement of 8-anilino-naphthalene-1-sulphonic acid fluorescence demonstrated by coupled membrane vesicles or proteoliposomes containing an 'outward facing' H⁺-ATPase (BF₀F₁ complex) was measured spectrofluorimetrically as previously described (Clarke & Morris, 1977b).

Assay of ATP synthetase activity

The assay procedure adopted was based on that described by Yoshida *et al.* (1975) for the H⁺-ATPase of thermophilic bacterium PS3.

Bacteriorhodopsin purified from suitably grown cultures of *Halobacterium halobium* R-1 (Oesterhelt & Stoekenius, 1974), was incorporated by the cholate-dialysis procedure (Yoshida *et al.*, 1975) into everted membrane vesicles or artificial proteoliposomes constructed with purified H⁺-ATPase (BF₀F₁ complex) and phospholipids extracted from vegetative-cell membranes of *Cl. pasteurianum* (Clarke & Morris, 1977b, 1979). Samples of suspensions of these bacteriorhodopsin-containing membrane vesicles (or proteoliposomes) containing 1–3 mg of protein were diluted to 1.4 ml with a solution containing 40 μ mol of Tris/acetate buffer, pH 7.8, 10 μ mol of sodium phosphate buffer, pH 7.8, 60 μ mol of glucose, 200 μ mol of sucrose, 0.2 μ mol of EDTA, 4 μ mol of MgSO₄, 200 μ mol of Na₂SO₄, 4 μ mol of ADP and 5 mg hexokinase [EC 2.7.1.1; 80 units (μ mol/min)]. Sodium [³²P]phosphate (20 μ Ci, carrier-free) was then added and the final mixture (2 ml) was illuminated under N₂ at 37°C by a 20 cm-distant intense light-source (supplied by a Kodak Carousel model S projector). Samples (0.5 ml) were removed at intervals and were rapidly mixed at 0°C with 0.1 ml of 50% (w/v) trichloroacetic acid. After centrifugation at 12000 g for 5 min at 2°C, portions (0.2 ml) of the deproteinized extract were mixed with 4 ml of 1.25 M-HClO₄, 1 ml of 5% (w/v) ammonium molybdate and 5 ml of a mixture (1:1, v/v) of 2-methylpropan-1-ol and benzene. After vigorous vortex mixing and phase separation (by standing at 0°C for 5 min), the upper (organic) phase was removed. The aqueous phase was serially extracted (at least four times) with 5 ml volumes of water-satu-

rated 2-methylpropan-1-ol until the upper phase was completely colourless. The radioactivity of the residual aqueous phase (attributable to glucose 6-[³²P]-phosphate) was then assayed. The ATP synthetase activity was calculated from the initial (linear) rate of production of glucose 6-[³²P]phosphate.

Measurement of radioactivity

Radioactivity was measured by liquid-scintillation counting in a Beckman LS 200B spectrometer (Beckman RIIC, High Wycombe, Bucks., U.K.). Aqueous samples (less than 0.5 ml) were assayed for radioactivity in 10 ml amounts of a mixture consisting of 4 g of 2,5-diphenyloxazole and 500 ml of Triton X-100 in toluene to a final volume of 1 litre.

Assay of protein

Protein was generally measured by the method of Lowry *et al.* (1951); a standard curve was prepared with crystalline bovine serum albumin (fraction V). Protein in column-chromatography eluates was continuously monitored by following absorbance at 278 nm and 260 nm during passage through a LKB Uvicord III detector fitted with a chart recorder (LKB Instruments, South Croydon, Surrey, U.K.).

SDS/polyacrylamide-gel electrophoresis

This was performed as previously described, with marker proteins of known molecular weights being simultaneously electrophoresed in adjacent gels (Clarke *et al.*, 1975). Gels were stained for protein with Coomassie Blue by the method of Fairbanks *et al.* (1971).

Chemicals and materials

ATP, 8-anilino-naphthalene-1-sulphonic acid, ATP, bovine serum albumin (crystalline), deoxyribonuclease (EC 3.1.4.5, 1115 Kunitz units/mg), NN'-dicyclohexylcarbodi-imide, hexokinase [EC 2.7.1.1, 16 units (μ mol/min)/mg], imidazole, lysozyme [EC 3.2.1.17, 20000 units (μ mol/min)/mg], sodium cholate, SDS, Tris, trypsin (EC 3.4.21.4, 11400 Bz-Arg-OEt units/mg) and trypsin inhibitor protein (from soya bean) were all purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. 2-Mercaptoethanol and all other chemicals were supplied by BDH Chemicals, Poole, Dorset, U.K. DEAE/Sephacel CL-6B, Sephadex G-200, Sephadex LH-20 and Sepharose CL-4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium [³²P]phosphate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Ultrafiltration membranes, i.e. Amicon PM10, Amicon UM2 and Centriflo cones, were purchased from Amicon, High Wycombe, Bucks., U.K.

Results

Trypsin activation of mother-cell-membrane ATP phosphohydrolase

Previous studies (Clarke & Morris, 1977a) had shown that the membrane ATP phosphohydrolase activity of *Cl. pasteurianum* decreased more than 10-fold early in sporulation. However, although the ATP phosphohydrolase activity of vegetative-cell membranes was not enhanced by brief treatment with trypsin, the mother-cell-membrane H^+ -ATPase responded to exposure to trypsin by a marked increase in its ATP phosphohydrolase activity (Fig. 1). The extent of this trypsin activation was found to depend on the pH at which the mother-cell mem-

Fig. 1. Effect of trypsin treatment on the ATP phosphohydrolase activities of cell membranes from vegetatively growing or sporulating *Cl. pasteurianum*

Vegetative-cell membranes (O) and mother-cell membranes (●) were obtained and exposed to trypsin at zero time as described in the Experimental section. At intervals during incubation at 37°C samples were removed into an excess of soya-bean trypsin-inhibitor protein and their ATP phosphohydrolase activities were immediately assayed.

brane fraction had been prepared. The activation was most marked when the membrane fragments had been isolated in buffers of pH 7.25–8.0 (Fig. 2) and was least obvious when the preparative buffer had a pH value less than pH 6.5. Mother-cell membranes prepared at pH 7.5 lost their capacity to respond to brief trypsin treatment by an increase in ATP phosphohydrolase activity when they were washed in buffers of pH value more acid than pH 6.5. On the assumption that this behaviour was explicable by the presence in the initial mother-cell-membrane H^+ -ATPase of a trypsin-sensitive inhibitory protein that dissociated from the complex at pH values more acid than pH 6.5, an attempt was made to exploit this property to isolate the inhibitory protein. Since it had also been noted that, even at pH 7.6, washing of the mother-cell membranes with buffer mixtures containing a high concentration of 2-mercaptoethanol diminished the response to trypsin treatment (Fig. 2b), the concentration of this thiol was decreased in the buffer mixtures employed in the protoplast-formation and -disruption steps of mother-cell-membrane isolation.

Purification of a trypsin-sensitive inhibitor protein from mother-cell membranes

Initially, sporulating cells of the parent wild-type organism (*Cl. pasteurianum* 6013) were exploited as the source of suitable mother-cell membranes. When, however, it was found that sporulating cells of the granulose-negative mutant strain (MR505) were an equally rich source of the inhibitor protein, this organism was thereafter preferred.

A mother-cell-membrane preparation (800 mg of protein) was obtained from a sporulating culture of *Cl. pasteurianum* harvested when over 90% of the organisms contained phase-dark forespores. The membrane fragments were washed in 90 mM-Tris/ H_2SO_4 buffer, pH 7.6, containing 6 mM- $MgSO_4$ plus 1 mM-ATP, and were resuspended in 27 ml of a mixture of 0.1 M-Tris plus 0.1 M-imidazole adjusted to pH 6.0 with H_2SO_4 and supplemented with 20 mM-2-mercaptoethanol, 6 mM- $MgSO_4$ and 1 mM-ATP. The suspension was stirred for 10 h at 4°C under O_2 -free N_2 , and the membranes were then sequentially washed in 27 ml volumes of the supplemented buffer mixture adjusted first to pH 8.5, then to pH 6.0 and finally again to pH 8.5. The washings were pooled with the first extract and clarified by centrifugation at 200 000 g for 30 min at 4°C. The resultant supernatant was concentrated to 5 ml by ultrafiltration under N_2 at 5°C through an Amicon PM10 membrane (10 000-mol.wt. exclusion). The ultrafiltrate was retained, and the concentrate was subjected to further ultrafiltration by centrifugation at 4000 g at 4°C through an Amicon Centriflo cone filter (50 000-mol.wt. exclusion). This centrifuged ultrafiltrate was pooled with the primary Amicon

Fig. 2. Extent of trypsin activation of mother-cell-membrane ATP phosphohydrolase is affected (a) by the pH of isolation of the membranes and (b) by prior exposure to 2-mercaptoethanol

(a) Sporulating cells of *Cl. pasteurianum* were converted into protoplasts, samples of which were suspended and ruptured in 0.1 M-Tris plus 0.1 M-imidazole with 6 mM-MgSO₄ and 1 mM-ATP adjusted to pH 6.0 (●), pH 7.0 (▲) or pH 7.5 (○) with H₂SO₄. After cells were ruptured by French-pressure-cell treatment, the membranes were recovered, washed once in buffer mixture of the same pH and finally resuspended in 90 mM-Tris/H₂SO₄ buffer, pH 7.6 containing 6 mM-MgSO₄ before measurement of the course of trypsin activation of their ATP phosphohydrolase activity (see the Experimental section). (b) After isolation in buffer mixture of pH 7.6, a mother-cell-membrane preparation was washed in the same buffer mixture with (■) or without (Δ) 20 mM-2-mercaptoethanol. The washed membranes were recovered and resuspended in pH 7.6 buffer mixture minus 2-mercaptoethanol for assay of the course of trypsin activation of their ATP phosphohydrolase activity.

PM10 ultrafiltrate and the whole was concentrated to 3 ml over an Amicon UM2 membrane (1000-mol.wt. exclusion). The concentrate was loaded on to a Sephadex G-200 column (15 mm × 350 mm) that had previously been equilibrated with 0.1 M-imidazole/H₂SO₄ buffer, pH 6.0, containing 6 mM-MgSO₄ plus 1 mM-ATP. On elution with this buffer mixture, most of the applied protein emerged in two peaks (Fig. 3). The (7 ml) eluate fractions containing protein were individually assayed for their ability to inhibit the ATP phosphohydrolase activity of a mixture of vegetative-cell membranes of *Cl. pasteurianum* that had been depleted of endogenous trypsin-sensitive inhibitor protein by prior washing with buffer mixture of pH 6.0 (see above). [When it transpired that the trypsin-sensitive inhibitor protein inhibited the ATP phosphohydrolase activity of the vegetative-cell membranes (below), this assay for the inhibitor protein in column-eluate fractions was performed with these membranes alone.]

The ATP phosphohydrolase-inhibitor activity was confined to the second (major) eluted peak of protein

Fig. 3. Gel filtration of trypsin-sensitive inhibitor protein through Sephadex G-200

Elution was at 10 ml/h and the emergent fractions (7 ml) were assayed for their contents of protein (—, A₂₇₈) and of trypsin-sensitive inhibitor protein (histogram) as described in the text.

Fig. 4. SDS/polyacrylamide-disc-gel electrophoresis of purified trypsin-sensitive inhibitor protein

The electrophoretogram was stained with Coomassie Blue and was scanned spectrophotometrically at 609 nm with a Gilford 2400-S recording spectrophotometer. Total protein applied was 100 μ g.

(Fig. 3). The eluate fractions comprising this peak were pooled and concentrated to a final volume of 3 ml over an Amicon UM2 ultrafiltration membrane. SDS/polyacrylamide-disc-gel electrophoresis of a sample of this preparation revealed the presence of a single protein component of mol.wt. $10000 \pm 10\%$ (Fig. 4). The total yield of purified trypsin-sensitive inhibitor protein was approx. 30 mg.

Effect of trypsin-sensitive inhibitor protein on the ATP phosphohydrolase activity of the vegetative-cell-membrane H^+ -ATPase

A great excess of trypsin-sensitive inhibitor protein virtually completely inhibited the initial ATP phosphohydrolase activity of a vegetative-cell-membrane preparation (Fig. 5a), but substantial activity was progressively recovered during the course of trypsin treatment, until after 60 min some 75% of the original enzyme activity was again displayed (Fig. 5b). Continued treatment with trypsin caused the same rapid destruction of enzyme activity as was provoked from the outset by trypsin treatment of the vegetative-cell-membrane ATPase unsupplemented with trypsin-sensitive inhibitor protein (Fig. 5b).

Fig. 5. Effects of purified trypsin-sensitive inhibitor protein on the ATP phosphohydrolase of vegetative-cell membranes of *Cl. pasteurianum*

(a) Inhibition of activity. Vegetative-cell membranes (0.25 mg of protein/ml) were incubated for 30 min at 37°C with various concentrations of trypsin-sensitive inhibitor protein (TSIP) before assay of their ATP phosphohydrolase activity. (b) Conferral of trypsin activatability. Suspensions of vegetative-cell membranes (0.5 mg of protein each) in 1 ml of 90 mM-Tris/ H_2SO_4 buffer, pH 7.6, containing 6 mM- $MgSO_4$ and 1 mM-ATP at 37°C were treated with trypsin (10 μ g) at time (2), without (O) or with (●) prior addition of 0.5 mg of trypsin-sensitive inhibitor protein at time (1). Serial samples (20 μ l) were removed for assay of ATP phosphohydrolase activity in the presence of soya-bean trypsin inhibitor protein (5 μ g/ml).

Artificial everted proteoliposomes constructed from purified vegetative-cell-membrane H⁺-ATPase (BF₀F₁ complex) plus a mixture of phospholipids demonstrated ATP-provoked enhancement of 8-anilino-naphthalene-1-sulphonic acid fluorescence (Clarke & Morris, 1977b). This response to ATP was almost completely abolished by prior incubation of the proteoliposomes (20 µg of protein) with a great excess (50 µg) of trypsin-sensitive inhibitor protein (Fig. 6). However, when such inhibitor-protein-treated proteoliposomes were first washed and then treated with trypsin, ATP-provoked enhancement of 8-anilino-naphthalene-1-sulphonic acid fluorescence could again be demonstrated (Fig. 6).

Fig. 6. Construction of trypsin-activatable H⁺-ATPase from trypsin-sensitive inhibitor protein plus vegetative-cell-membrane ATP phosphohydrolase of *Cl. pasteurianum*

Artificial everted proteoliposomes constructed from purified vegetative-cell-membrane ATPase (BF₀F₁ complex) plus a mixture of phospholipids (Clarke & Morris, 1977b) demonstrated ATP-provoked enhancement of 8-anilino-naphthalene-1-sulphonic acid (ANS) fluorescence. (a) Proteoliposome suspension (20 µg of protein) treated first with 8-anilino-naphthalene-1-sulphonic acid (33 µM) and then with ATP (18 mM). Fluorescence emission at 470 nm was followed at room temperature during excitation at 370 nm. (b) As (a), but after prior incubation for 15 min at 37°C of proteoliposomes (20 µg of protein) with trypsin-sensitive inhibitor protein (50 µg). (c) As (b), but trypsin-sensitive inhibitor protein was first removed by washing the inhibitor-protein-treated proteoliposomes with 90 mM-Tris/H₂SO₄ buffer, pH 7.6, containing 1 mM-MgSO₄ followed by exposure to trypsin (1 µg) for 10 min at 37°C.

Effect of trypsin-sensitive inhibitor protein on the ATP synthetase activity of the vegetative-cell-membrane H⁺-ATPase

With purified bacteriorhodopsin incorporated into crude everted membrane vesicles derived from vegetative-cell membranes of *Cl. pasteurianum*, light-dependent synthesis of ATP from ADP plus P_i could be demonstrated when the ATP produced by the membrane H⁺-ATPase was trapped by the action of hexokinase (Clarke & Morris, 1979). The vegetative-cell-membrane ATPase displayed considerably less ATP synthetase activity than did similarly assayed preparations of crude mother-cell-membrane ATPase (154 pmol of ATP synthesized/min per mg of protein compared with 260 pmol/min per mg). However, after preincubation of bacteriorhodopsin-containing vegetative-cell-membrane vesicles (1 mg of protein) with trypsin-sensitive inhibitor protein (0.25 mg of protein), and enhanced rate of ATP synthesis was measured (263 pmol/min per mg of membrane protein), which resembled that shown by the mother-cell-membrane vesicles.

When the light-plus-bacteriorhodopsin-driven ATP synthetase activities of cell-membrane preparations were assayed with membrane vesicles prepared from organisms harvested at different stages of culture growth and sporulation, it was found that the specific ATP synthetase activity of the cell membrane increased markedly early in sporulation (Fig. 7). This increase approximately coincided with the decrease in the specific ATP phosphohydrolase activity of the membrane H⁺-ATPase and its acquisition of susceptibility to trypsin activation in this hydrolytic mode (Clarke & Morris, 1977a). After Stage III of sporulation (marked by the appearance of phase-dark forespores) the membrane-associated ATP synthetase activity degenerated quite rapidly. The specific activity of the trypsin-activated ATP phosphohydrolase of the mother-cell membrane similarly, but more slowly, decreased at this time. Vesicles made from mother-cell-membrane fragments isolated at this and subsequent stages of sporulation were incapable of demonstrating any energy-coupled response such as ATP-dependent enhancement of 8-anilino-naphthalene-1-sulphonic acid fluorescence or ATP-provoked proton uptake.

Discussion

A notable feature of the purified mother-cell-membrane H⁺-ATPase (BF₀F₁ complex) of sporulating *Cl. pasteurianum* was the presence in quite large quantities of a protein (subunit *e*) that was not found in similarly purified H⁺-ATPase preparations from vegetative-cell membranes of this organism (Clarke & Morris, 1977a; Clarke *et al.*, 1979). The trypsin-sensitive inhibitor protein that was purified from mother-cell membranes migrated identically with

Fig. 7. Cell-membrane H^+ -ATPase of *Cl. pasteurianum* during late-exponential growth and sporulation

(a) Course of sporulation in batch culture of *Cl. pasteurianum* indicating state of organisms harvested at designated times as sources of the cell-membrane preparations assayed in (b). Cell number (●), intracellular deposition of granulose (○) and appearance of phase-dark forespores (▲) and of phase-bright spores (△) were monitored during anaerobic incubation at 37°C. (b) Preparations of cell membrane were assayed as described in the text, for their specific ATP synthetase activity (▨) and their specific ATP phosphohydrolase activity before (■) and after (□) brief treatment with trypsin. The membrane preparations were derived from organisms harvested at the corresponding times in the course of sporulation of the batch culture (as indicated in a).

membrane ATP phosphohydrolase is attributable to the preferential tryptic digestion of this subunit *e* of the mother-cell-membrane H^+ -ATPase. Though the dissociation *in vitro* of trypsin-sensitive inhibitor protein from the mother-cell membrane at pH 6.0 proved useful in its purification, until we know more of the binding characteristics of the inhibitor protein (i.e. the location of subunit *e* in the ATPase complex and its relations with the other subunits) it remains uncertain whether it is so readily dissociable from the complex *in vivo*, when the internal pH of the mother cell might well attain a pH more acid than pH 6.5 (Riebeling *et al.*, 1975). Yet it was gratifying that purified trypsin-sensitive inhibitor protein would interact with the vegetative-cell-membrane H^+ -ATPase complex to diminish its ATP phosphohydrolase activity and render the residual activity enhanceable by trypsin treatment in the manner of the mother-cell-membrane H^+ -ATPase. That addition of trypsin-sensitive inhibitor protein also increased the 'forced' ATP synthetase activity of the vegetative-cell-membrane H^+ -ATPase was particularly interesting in view of reports that a trypsin-sensitive inhibitor protein is commonly associated with bacterial ATPases that normally function as ATP synthetases (West, 1974). Indeed, it has been proposed that in such enzyme complexes the trypsin-sensitive inhibitor protein helpfully serves to favour the ATP synthetase function by inhibiting consumption of the product by reverse ATP phosphohydrolysis (Nelson, 1976; Racker, 1976; Pedersen, 1977).

We do not suggest that the acquisition of a trypsin-sensitive inhibitor protein by the H^+ -ATPase of sporulating cells of *Cl. pasteurianum* indicates a change in function of the mother-cell enzyme (i.e. from ATP phosphohydrolase to ATP synthetase). It may be that the trypsin-sensitive inhibitor protein could serve to control the ATP phosphohydrolase activity of the enzyme (in a manner possibly responsive to changes in internal pH). Alternatively, its formation could be wholly fortuitous; it could conceivably be a by-product of specific proteolysis associated with the sporulation programme and the cause of concurrent changes in certain other of the protein subunits of the ATPase complex (Clarke & Morris, 1977a). Whether trypsin-sensitive inhibitor protein has its origin in a pre-existing protein or is synthesized *de novo* during sporulation requires investigation. It certainly does not appear to be an artifact of generalized proteolysis during isolation of the mother-cell membranes, since addition of inhibitors of proteolysis (e.g. EDTA, phenylmethanesulphonyl fluoride) during protoplast breakage and membrane purification did not diminish the yield of trypsin-sensitive inhibitor protein or change the polypeptide composition of the mother-cell-membrane H^+ -ATPase complex (Clarke, 1978). Furthermore,

this novel subunit on SDS/polyacrylamide-gel electrophoresis, and it is reasonable to suppose that the trypsin activation displayed by the mother-cell-

strong circumstantial evidence for sporulation-associated change *in vivo* in the constitution of *Cl. pasteurianum* cell-membrane H⁺-ATPase is provided by the acquisition by sporulating organisms of resistance to the bacteriocin butyricin 7423, which *in vitro* inhibits the vegetative-cell-membrane H⁺-ATPase but not the mother-cell-membrane H⁺-ATPase (Clarke & Morris, 1977a).

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References

- Booth, I. R. & Morris, J. G. (1975) *FEBS Lett.* **59**, 153–157
- Clarke, D. J. (1978) Ph.D. Thesis, University of Wales
- Clarke, D. J. & Morris, J. G. (1976) *Biochem. J.* **154**, 725–729
- Clarke, D. J. & Morris, J. G. (1977a) in *Spore Research 1976* (Barker, A. N., Wolf, J., Ellar, D. J., Dring, G. J. & Gould, G. W., eds), pp. 359–372, Academic Press, London
- Clarke, D. J. & Morris, J. G. (1977b) *Biochem. Soc. Trans.* **5**, 140–143
- Clarke, D. J. & Morris, J. G. (1979) *Eur. J. Biochem.* **98**, 613–620
- Clarke, D. J., Robson, R. M. & Morris, J. G. (1975) *Antimicrob. Agents Chemother.* **7**, 256–264
- Clarke, D. J., Fuller, F. M. & Morris, J. G. (1979) *Eur. J. Biochem.* **98**, 597–612
- Fairbanks, G., Steck, T. C. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Lloyd, D. & Edwards, S. W. (1976) *Biochem. J.* **160**, 335–342
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mackey, B. M. & Morris, J. G. (1971) *J. Gen. Microbiol.* **66**, 1–13
- Morris, J. G. (1977) *Trends Biochem. Sci.* **2**, 81–83
- Muñoz, E., Freer, J. H., Ellar, D. J. & Salton, M. J. R. (1968) *Biochim. Biophys. Acta* **150**, 531–537
- Nelson, N. (1976) *Biochim. Biophys. Acta* **456**, 314–338
- Oesterhelt, D. & Stoebenius, W. (1974) *Methods Enzymol.* **31**, 667–678
- Pedersen, P. L. (1977) *J. Bioenerg.* **6**, 243–275
- Racker, E. (1976) *A New Look at Mechanisms in Bioenergetics*, p. 43, Academic Press, New York
- Riebeling, V., Thauer, R. K. & Jungermann, K. (1975) *Eur. J. Biochem.* **55**, 445–453
- Robson, R. L., Robson, R. M. & Morris, J. G. (1974) *Biochem. J.* **144**, 503–511
- West, I. C. (1974) *Biochem. Soc. Spec. Publ.* **4**, 27–38
- Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., Takeuchi, Y. & Ohno, K. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1295–1300