Biochemical Evidence for the Reversed Polarity of the Outer Membrane of the Bacterial Forespore

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1. Measurement of certain membrane-bound enzymic activities was used to study the orientation of the outer membrane of the double-membraned forespore of *Bacillus megaterium* KM. 2. Adenosine triphosphatase, NADH dehydrogenase and L-malate dehydrogenase, NADH oxidase and L-malate oxidase were virtually undetectable in intact protoplasts, but were readily detected in intact stage III or IV forespores, consistent with reversed polarity of the outer forespore membrane relative to the mother-cell plasma membrane. 3. Measurement of NADH oxidase activity revealed that intact stage III forespores had the same high affinity for NADH as protoplast-membrane preparations and protoplast lysates, consistent with ready access of NADH to oxidation sites on the outer forespore membrane. 4. Forespores and protoplasts showed osmometric behaviour in solutions of non-permeant solutes consistent with the presence of an intact permeability barrier in these structures.

Bacterial endospores are unusual in many respects, notably in their resistance to heat, radiation and bactericidal agents, and for their lack of detectable metabolism. Such spores may remain dormant for considerable periods and yet retain the capacity to germinate and resume vegetative growth within minutes after exposure to solutions of appropriate germinants. Many aspects of spore composition and structure have been investigated in an attempt to discover the biochemical basis for these properties. One feature of spores which distinguishes them from all other bacterial cells is the presence of a double membrane surrounding the spore cytoplasm. (Here it is assumed that the outer layer of Gram-negative bacteria is not analogous to a plasma membrane.) These two membranes arise as a consequence of invagination of the vegetative cell plasma membrane, which is an early event in spore formation (Fig. 1). This invagination results first in the formation of a membrane septum asymmetrically disposed towards one cell pole to produce two cellular compartments. The smaller compartment (forespore) is destined to become the mature spore and in the subsequent stages of sporulation this compartment is engulfed within the larger compartment (mother cell) by a continued proliferation of the initial membrane septum towards the cell pole. After engulfment is complete, the forespore exists within the mother-cell cytoplasm as a discrete cell containing at least one complete genome and bounded by a double membrane (Fig. 1). This description of the initial stages of sporulation is based on the electron-microscopic studies of Young & Fitz-James (1959*a,b,c*) with

Bacillus cereus and is similar in other *Bacillus* species (Ellar *et al.*, 1967).

Although the existence of a double membrane around the developing spore is in itself unusual, the orientation of the membrane surface involved is perhaps of even greater importance in considering the biosyntheses and transport mechanisms that are an essential feature of spore morphogenesis. From the nature of the membrane invagination which gives rise to the spore membranes it appears that the outer of the two membranes has reversed surface polarity with reference both to the inner spore membrane and the mother-cell plasma membrane, i.e. the surface that originated as the inner surface of the mother-cell plasma membrane remains apposed to the spore cytoplasm. Fig. 1 shows that by the nature of the engulfment process the outer surface of the spore outer membrane arises from what was previously the inner (cytoplasmic) surface of the mother-cell plasma membrane. In the double membrane of the completed forespore it follows that what were previously the outer surfaces of the mother-cell membrane now face each other. This apparent reversal of normal membrane polarity must be taken into account when considering the transport of metabolites and ions to and from the forespore compartment during spore morphogenesis (Murrell, 1967; Hanson *et al.*, 1970). For example, if each of the forespore membranes retained the normal mechanisms for active transport, these would apparently operate in opposite directions (Freese, 1972).

Since the early work of Mitchell & Moyle (Mitchell

Fig. 1. Formation of the bacterial forespore showing the 'sidedness' of the membranes involved

Abbreviations: N, nuclear material; pm, plasma membrane; cw, cell wall; ofm, outer forespore membrane; ifm, inner forespore membrane. In the two insets in the middle of the diagram the symbols O and I represent the 'sidedness' of the membranes; O represents the outer surface of the plasma membrane, I the inner surface. For details of the invagination sequence see the text.

& Moyle, 1956; Mitchell, 1962) it has been known that the intact plasma membrane of bacteria is impermeable to a range of hydrophilic solutes including ATP and NADH. These observations were used experimentally to establish that certain membrane-bound enzymes are located on the inner surface of the bacterial membrane. With the development of a system for isolating forespores from the mother cell at all stages of sporulation, it has now become possible to use a similar experimental approach to investigate this possibility of reversed membrane polarity in the intact forespore.

Wilkinson & Ellar (1975) showed that membranes from exponentially growing vegetative cells, forespores and mother cells will oxidize NADH and L-malate (EC 1.1.3.3) and possess ATPase* activity (EC 3.6.1.3). Consequently in these experiments the oxidation of NADH and L-malate by vegetative protoplasts, sporulating cell protoplasts and forespores was compared by using the oxygen electrode. In addition, these intact cell types, together with the membranes prepared from them by lysis, were examined by direct enzyme assay of NADH dehydrogenase (EC 1.6.99.3) and L-malate dehydrogenase (EC 1.1.1.37) and of ATPase. As a control, the intactness of forespores and protoplasts was confirmed by observing their osmometric behaviour

* Abbreviation: ATPase, adenosine triphosphatase.

when suspended in solutions of permeant and non-permeant solutes. These results provide direct biochemical evidence that the outer forespore membrane has the reversed orientation predicted from morphological observations.

Experimental

Materials

The organism used was a sporogenic strain of *Bacillus megaterium* KM that remains lysozyme-sensitive throughout sporulation and was cultivated synchronously as described by Ellar & Posgate (1974). NADH, ATP and DL- α -glycerol phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); other reagents were of the highest grade commercially available.

Methods

Preparation of protoplasts. Protoplasts that were to be used in studies with the oxygen electrode, and for studying the osmometric behaviour of protoplasts and forespores, were made by lysozyme (200 μ g/ml) treatment of washed organisms in 0.1M-KH₂PO₄ buffer adjusted to pH 6.3 with 5M-NaOH, containing 0.6M-sucrose and 16mM-MgSO₄ (SPM buffer, pH 6.3) at 37°C as described by Ellar & Posgate (1974).

Protoplasts that were to be used for ATPase and dehydrogenase assays were prepared from washed organisms by treatment with lysozyme (200 µg/ml) in 0.1M-Tris-HCl buffer, pH 7.2, containing 0.6M-sucrose and 8mM-CaCl₂, and were washed in lysozyme-free buffer. In the present studies organisms were harvested and washed at room temperature (20°C), and protoplasts and forespores were prepared and kept at room temperature. Cooling tended to make protoplasts more susceptible to lysis during routine handling. In addition the ATPase of this strain of *B. megaterium* is known to be cold labile (J. A. Deans, unpublished work), like that described by Mirsky & Barlow (1971) for another strain of *B. megaterium*.

Isolation of forespores. Forespores were released from protoplasts by selective rupture of the mother-cell membrane by mild sonication and were isolated by differential centrifugation essentially as described by Ellar & Posgate (1974) and Eaton & Ellar (1974). After protoplasts were formed the suspension was sonicated for up to 6 × 1 s pulses with a 1.2 cm (0.5 in) sonic probe (Dawe Instruments Ltd., London W.3, U.K.) operating at maximum output at room temperature (20°C). The released forespores were separated from mother-cell material by centrifugation at 11 600g for 3 min at 20°C. Forespores that were to be used in oxidase assays and osmometric studies were washed twice by resuspension and centrifugation in 0.1M-KH₂PO₄ buffer, pH 7.5, containing 0.6M-sucrose and 16mM-MgSO₄ (SPM buffer, pH 7.5) and resuspended in a small volume of this medium. Forespores that were to be used in ATPase and dehydrogenase assays were washed in the Tris-sucrose-CaCl₂ buffer used for lysozyme treatment.

Sonic disruption of protoplasts and forespores. For detection of ATPase and L-malate dehydrogenase activities protoplasts were disrupted under the same sonication conditions as described above for the release of forespores. Protoplast disruption was monitored by observation in the light microscope after each few sonic pulses. Forespore suspensions were disrupted by sonication under the same conditions except that up to 20 × 1 s pulses had to be used to disrupt the double-membraned forespores. The progress of forespore disruption was monitored microscopically.

Isolation of membrane and cytoplasmic fractions of protoplasts and forespores. For ATPase and dehydrogenase assays, vegetative cell membranes and forespore membranes were prepared by diluting protoplast and forespore suspensions respectively in 0.05M-Tris-HCl buffer, pH 7.2, containing 8mM-CaCl₂; membranes were recovered by centrifugation at 105 000g for 1 h. The supernatant was regarded as the cytoplasmic fraction. Mother-cell membranes were recovered from disrupted stage III and IV

sporangial protoplasts by centrifugation at 105 000g for 1 h after removal of the intact forespores by lower-speed centrifugation (Ellar & Posgate, 1974). The 105 000g supernatant constitutes the mother-cell cytoplasm. Vegetative-cell membranes for determination of the K_m values of NADH oxidation were prepared by lysozyme treatment of late-exponential-phase organisms and lysis in 0.05M-Tris-HCl, pH 7.5, as described by Wilkinson & Ellar (1975).

Measurement of oxidase activities. O₂ consumption by various preparations was measured with an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) at 30°C. Reactions with intact protoplasts and forespores were carried out in SPM buffer, pH 7.5. Protoplasts and forespores were lysed by the addition of a small volume (up to 0.2 ml) of concentrated protoplast or forespore suspension in SPM buffer to 0.05M-KH₂PO₄ buffer, pH 7.5 (final vol. 3 ml), in the reaction vessel of the oxygen electrode. Substrates were dissolved in 0.05M-Tris-HCl buffer, pH 7.5, containing 0.6M-sucrose and were used at a final concentration of 10 mM, except for NADH which was 1 mM. With intact protoplasts and forespores the rate of O₂ uptake was measured for about 3 min in the absence of substrate in the SPM buffer, pH 7.5 (endogenous respiration), before the substrate to be tested was added. The O₂ uptake of protoplast and forespore lysates in 0.05M-phosphate buffer was measured for a similar period before the addition of substrate. Membrane NADH oxidase activities were also measured in 0.05M-KH₂PO₄ buffer, pH 7.5.

Enzyme assays. (a) NADH dehydrogenase. This was assayed as described by Ellar *et al.* (1971) except that the reaction was carried out in the presence of 0.1M-Tris-HCl buffer, pH 7.5, containing 0.6M-sucrose and 8mM-CaCl₂, instead of 0.05M-Tris-HCl, pH 7.5. Reactants were made up in the Tris-sucrose-CaCl₂ buffer.

(b) L-Malate dehydrogenase. This was assayed as described above for NADH dehydrogenase by substituting L-malate (final concn. 25 mM) for NADH in the assay.

(c) ATPase. This was assayed by the method described by Muñoz *et al.* (1968), except that the reaction was carried out in 0.1M-Tris-HCl buffer, pH 7.5, containing 0.6M-sucrose and 8mM-CaCl₂.

Measurement of protein concentration. The method of Lowry *et al.* (1951) was used, with 0.1 ml of 10% (w/v) sodium dodecyl sulphate included in the assay to aid solubilization of preparations. Bovine serum albumin was used as the standard.

Observation of the osmometric behaviour of protoplasts and forespores. Protoplasts and forespores prepared as described above were washed and resuspended in 0.1M-KH₂PO₄ buffer, pH 6.3, containing 0.3M-sucrose. The suspension density was adjusted so that a 30-fold dilution in sucrose buffer gave an absorbance of approx. 0.3 at 600 nm. Samples

(0.1 ml) of protoplasts or forespores were then added to 2.9 ml of 0.1M-KH₂PO₄ buffer containing sufficient sucrose, glycerol or NaCl to give the required final solute concentration. The mixture was shaken and the absorbance at 600nm read in a Unicam SP.500 spectrophotometer (Pye-Unicam Ltd., Cambridge, U.K.) after 10min. All solutions used were at room temperature. Absorbance values obtained were corrected for changes in refractive index of the medium caused by the added solute in the case of sucrose (Knowles, 1971).

Results

Comparison of the ATPase activity of intact protoplasts and forespores and its distribution in disrupted protoplasts and forespores

When intact vegetative-cell protoplasts or stage III or IV protoplasts were assayed for ATPase, no activity was detected (Table 1). When protoplasts were disrupted by mild sonication ATPase activity was readily detectable. Fractionation of the disrupted protoplasts into membrane and cytoplasm revealed that 75% of the total ATPase activity was associated with the membrane. The small percentage of ATPase activity present in the cytoplasmic fraction was possibly due to the presence of small membrane fragments produced by sonication, not deposited by the centrifugation conditions used. In marked contrast with intact protoplasts, intact forespores showed significant ATPase activity. This activity was approximately doubled after sonication of the forespores. These results are consistent with ready access of ATP to an outer forespore membrane in intact forespores, and to both inner and outer forespore-

membranes after sonication. Virtually all of the forespore ATPase activity was recovered in the membrane fraction.

Comparison of the L-malate dehydrogenase and NADH dehydrogenase activities in intact protoplasts and forespores and their distribution in disrupted protoplasts and forespores

Examination of fractionated protoplasts and forespores (Table 2) revealed that L-malate dehydrogenase was entirely associated with the membrane fraction of both protoplasts and forespores. The addition of L-malate to suspensions of intact protoplasts did not stimulate dehydrogenase activity over the endogenous rate. On sonic disruption of the protoplasts, endogenous dehydrogenase activity fell substantially and L-malate dehydrogenase activity was readily detected. These observations are consistent with the localization of L-malate dehydrogenase on the inner surface of the plasma membrane. When L-malate was added to suspensions of intact forespores there was a marked stimulation of dehydrogenase activity over the endogenous rate. Unlike the ATPase activity, no marked increase in specific activity of L-malate dehydrogenase occurred on disruption of the forespores by mild sonication. It

Table 1. *Comparison of the ATPase activity of intact B. megaterium KM protoplasts and forespores and of the membranes isolated from these structures*

Protoplasts and forespores were prepared and disrupted and membranes were recovered as described in the Experimental section. ATPase activity was measured as described in the Experimental section. The experimental values represent the average of several batches of preparations from each growth stage.

Preparation Growth stage ...	ATPase activity (μ mol of P _i liberated/10min per mg of protein)		
	Vegetative	III	IV
Intact protoplast	0	0	0
Sonicated mother cell	0.61	0.19	0.45
Mother-cell membrane	4.27	0.72	0.98
Intact forespore	—	0.32	0.47
Sonicated forespore	—	0.63	0.87
Forespore membranes	—	1.12	1.03

Table 2. *Comparison of the L-malate dehydrogenase activity of intact B. megaterium KM protoplasts and forespores and of the membranes isolated from these structures*

Protoplasts and forespores were prepared and disrupted and membranes were recovered as described in the Experimental section. L-Malate dehydrogenase activity was measured as described in the Experimental section. The experimental values represent the average of several batches of preparations from each growth stage. The rate of 2,6-dichlorophenol-indophenol reduction in the absence of substrate has been subtracted from the values shown for intact protoplasts and forespores. Typical values were 60 and 25 nmol of 2,6-dichlorophenol-indophenol reduced/min per mg of protein for intact protoplasts and forespores respectively. Sonicated protoplasts and forespores and isolated membranes had essentially no endogenous activity.

Preparation Growth stage ...	L-Malate dehydrogenase activity (nmol of 2,6-dichloro- phenol-indophenol reduced/ min per mg of protein)		
	Vegetative	III	IV
Intact protoplast	0	0	0
Sonicated mother cell	21	19	16
Mother-cell membrane	65	109	103
Intact forespore	—	22	24
Sonicated forespore	—	21	28
Forespore membranes	—	34	52

was noted that forespore membranes had between 30 and 50% of the specific activity of the mother-cell membrane.

NADH dehydrogenase activity was detected in intact forespores but not in intact protoplasts (Table 3), which is consistent with the findings for ATPase and L-malate dehydrogenase activities.

Studies of the oxidative activities of protoplasts and forespores in the oxygen electrode

The oxidative activities of intact *B. megaterium* protoplasts in SPM buffer, pH7.5, were measured by using an oxygen electrode and compared with those of isolated, intact stage III and IV forespores. Intact late-exponential-phase protoplasts showed significant O₂ uptake in the absence of substrates, presumably through use of endogenous metabolites (Table 4). The addition of NADH, L-malate or DL- α -glycerol phosphate to the protoplast suspensions did not stimulate O₂ consumption, indicating that these substrates are not readily accessible to the electron-transport chain. However, the addition of a suitable quantity of the nutrient stock solution that is added to the sporulation medium (Ellar & Posgate, 1974), stimulated O₂ uptake, which is consistent with the ready access and metabolism of these nutrients which are required during the growth of the organisms. Intact protoplasts had similar O₂ consumption rates to the intact cells from which they were prepared. When the protoplasts were lysed in 0.05M-KH₂PO₄ buffer, pH7.5, no endogenous O₂ uptake was detected, as was noted with lysates of *Bacillus subtilis* protoplasts (Smith, 1962). This may be due to considerable dilution of the cellular contents on lysis. The loss of endogenous oxidative activity on protoplast lysis also constitutes evidence for the structural intactness of protoplasts in buffered sucrose medium. NADH, L-malate and DL- α -glycerol phosphate were readily oxidized by lysed protoplasts. Similar responses to NADH and L-malate were obtained with

protoplasts of organisms in stage III and IV of sporulation.

Stage III and IV forespores also showed considerable endogenous O₂ uptake, but a significant stimulation was observed on the addition of L-malate or NADH, indicating a ready access of these substrates to the electron-transport chain, as would be expected if the outer forespore membrane had reversed orientation (Table 4). Endogenous respiration ceased on lysis of stage III and IV forespores in 0.05M-phosphate buffer, which was indicative of the intactness of these structures; NADH and L-malate were readily oxidized by the lysate. DL- α -Glycerol phosphate was not oxidized by intact forespores or their lysates, or by intact stage III and IV protoplasts and their lysates. DL- α -Glycerol phosphate oxidase activity is, however, known to disappear from membranes of this organism during sporulation (Wilkinson & Ellar, 1975).

As a routine protoplasts were prepared and maintained in SPM buffer, pH 6.3, plus lysozyme (200 μ g/ml). When forespores were washed in SPM buffer, pH 6.3, and maintained in this buffer in the presence

Table 3. Comparison of the NADH dehydrogenase activity of intact *B. megaterium* KM protoplasts and forespores and of the membranes isolated from these structures

Experimental details are as for Table 2 except that NADH rather than L-malate was used as the substrate in the assay.

Preparation	NADH dehydrogenase activity (μ mol of 2,6-dichlorophenol- indophenol reduced/min per mg of protein)	
	III	IV
Intact protoplast	0	0
Mother-cell membrane	0.35	0.42
Intact forespore	0.30	0.22
Forespore membranes	0.69	0.60

Table 4. Comparison of the oxidative activities of *B. megaterium* KM protoplasts and forespores

Oxidative activities of intact protoplasts and forespores were measured in SPM buffer, pH 7.5, and oxidative activities of protoplast and forespore lysates were measured in 0.05M-KH₂PO₄ buffer, pH 7.5, as described in the Experimental section. The experimental values represent the average of several batches of preparations from each growth stage.

Growth stage	O ₂ consumption (nmol of O ₂ /min per mg of protein)									
	Vegetative		III				IV			
	Protoplasts		Protoplasts		Forespores		Protoplasts		Forespores	
	Intact	Lysed	Intact	Lysed	Intact	Lysed	Intact	Lysed	Intact	Lysed
None	142	0	179	0	56	0	157	0	28	0
NADH	142	456	179	227	113	231	157	225	60	160
L-Malate	142	29	179	29	65	30	157	36	33	27

Fig. 2. Lineweaver-Burk plots of NADH oxidation by vegetative cell membranes, vegetative cell lysates and intact stage III forespores of *B. megaterium* KM

Rates of NADH oxidation were measured in the oxygen electrode as described in the Experimental section. (a) NADH oxidation by vegetative cell membranes. Membranes were present at a concentration of 0.063 mg of protein per 3 ml of 0.05 M-KH₂PO₄ buffer, pH 7.5, reaction medium. (b) NADH oxidation by vegetative-cell protoplast lysates. Lysate was present at a concentration of 0.32 mg of protein per 3 ml of 0.05 M-KH₂PO₄ buffer, pH 7.5, reaction medium. (c) NADH oxidation by intact stage III forespores. Forespores were present at a concentration of 0.96 mg of protein per 3 ml of SPM buffer, pH 7.5, reaction medium. The endogenous O₂ uptake rate was subtracted from the NADH-stimulated rate.

of lysozyme (200 µg/ml), they showed exactly the same response to NADH and L-malate as forespores prepared from the same batch of cells, which had been washed and maintained in SPM buffer, pH 7.5, in the absence of lysozyme. Thus the lack of stimulation of O₂ consumption of protoplasts by NADH and L-malate is unlikely to be due to the fact that they are maintained in SPM buffer, pH 6.3, plus lysozyme before use. Sucrose added to a final concentration of 0.6 M to lysates in the oxygen-electrode reaction vessel showed no inhibitory effect on NADH- or L-malate-stimulated O₂ uptake.

Comparison of the affinity of intact forespores, vegetative-cell membranes and vegetative protoplast lysates for NADH oxidation

Hampton & Freese (1974) reported biphasic Lineweaver-Burk plots of NADH oxidation by membrane vesicles prepared from *Bacillus subtilis* by using lysozyme. The higher K_m value corresponded to the single K_m for NADH oxidation observed in whole cells, and was thought to be due to rate-limited entry of NADH into vesicles with the same orientation as in whole cells. The low K_m value was ascribed to the presence of a small portion of open or inverted vesicles in the preparations. These observations led us to predict that intact forespores should have a similar, high affinity for NADH as open protoplast membrane preparations, if the outer forespore membrane has a reversed orientation (see Fig. 1). Lineweaver-Burk plots (Fig. 2) of NADH oxidation by intact forespores were linear and yielded K_m values in the 30–60 µM range. These values compared favourably with protoplast membrane preparations

(35–50 µM) and with protoplast lysates (35–50 µM) (Fig. 2). These experiments further support the suggestion that the outer forespore membrane has a reversed orientation relative to the mother-cell plasma membrane, and argue against the stimulation of O₂ consumption of forespores by NADH being due to diffusion of NADH into the forespore.

Osmometric properties of protoplasts and forespores: evidence for the presence of an intact permeability barrier

Mitchell & Moyle (1956) showed that when intact Gram-negative bacteria or protoplasts were suspended in solutions of non-permeant solutes of different osmolality, the bacteria or protoplasts decreased in volume with increasing osmotic pressure of the medium. The smaller particles scattered more light and hence the extinction of the suspension increased with increasing osmotic pressure. These observations are consistent with the presence of an intact permeability barrier surrounding the protoplast.

To show that forespores retained an intact permeability barrier, we compared the changes in extinction of suspensions of protoplasts and forespores in solutions of different osmolality. Intact protoplast suspensions increased in extinction with increasing osmotic pressure of solutions of the non-permeant solutes NaCl and sucrose (Fig. 3). As expected the protoplasts were rapidly lysed in solutions of glycerol, a readily permeant solute (Fig. 3). In a similar manner intact forespore suspensions increased in extinction in solutions of increasing concentrations of sucrose and NaCl, and were readily lysed in solutions of glycerol (Fig. 4), thus providing evidence

Fig. 3. Changes in extinction of *B. megaterium* KM protoplast suspensions in solutions of different osmotic pressure

Late-exponential-phase protoplasts were prepared and their swelling and shrinking measured as described in the Experimental section. ○, Sucrose; △, NaCl; □, glycerol.

for the retention of an intact permeability barrier in forespores isolated by the method of Ellar & Posgate (1974). The magnitude of change in extinction was less in forespore suspensions. This may be associated with the fact that forespores are smaller structures surrounded by a double membrane, and with less capacity for expansion and contraction.

However, these observations do not in themselves eliminate the possibility that only the forespore inner membrane remains intact to serve as this permeability barrier. If the outer forespore membrane were to be damaged selectively during forespore isolation, the results of the substrate-accessibility experiments could equally well be interpreted as indicating that the outer forespore membrane has the same orientation as the mother-cell plasma membrane. For several reasons such selective damage does not appear to be occurring. First, no plasmolysis of the forespores was observed by phase microscopy when they were suspended in solutions of hyperosmotic sucrose and NaCl. If the outer forespore membrane had ceased to act as an osmotic barrier, leaving this role to be maintained by the inner membrane, the resulting plasmolysis would have been visible microscopically (Knowles, 1971). Secondly, electron microscopy of thin sections of forespores suspended before and during fixation in buffer containing 0.6M-sucrose

Fig. 4. Changes in extinction of *B. megaterium* KM stage III forespore suspensions in solutions of different osmotic pressure

Intact stage III forespores were prepared and their swelling and shrinking measured as described in the Experimental section. Symbols are as in Fig. 3.

confirmed that they were surrounded by a continuous double membrane and that no plasmolysis had occurred. If the outer forespore membrane no longer constituted an osmotic barrier, plasmolysis and contraction of the spore protoplast would have been observed, since the concentration of sucrose in the suspending buffer was such as to produce maximum forespore shrinkage (Fig. 4).

A further point which must be considered in evaluating these results is the possibility that the enzyme activities measured with intact forespores are the result of contamination of these preparations by residual fragments of mother-cell membrane. Calculations in which the enzyme specific activities (Tables 1, 2 and 3) were compared with data on the proportion of total cell protein represented by forespores and by mother-cell membrane revealed that no such contamination is occurring. In this connexion it has been established that at stage III the intact forespore and mother-cell membrane account for approx. 15% (Eaton & Ellar, 1974) and 10–15% respectively of the total cell protein. It is therefore possible to calculate the extent of contamination by mother-cell membrane which would be required to give the measured specific activities.

It is assumed for the purposes of these calculations that intact stage III forespores have no detectable enzymic activities and that the preparations were started from intact sporangial protoplasts containing 100mg of protein. Thus the 15mg (protein) of intact forespores derived from the above sporangial protoplast preparations would contain $15 \times 0.30 = 4.5$ units of NADH dehydrogenase activity (Table 3)

(here 1 unit is the amount of enzyme that transforms $1\mu\text{mol}$ of substrate/min). By using the specific activity of the mother-cell membrane it can be calculated that 12.8mg of mother-cell membrane would be needed to be present in the forespore fraction to account for the observed specific activity, i.e. an 85% contamination of the forespore preparation by mother-cell membrane. Similarly the ATPase and L-malate dehydrogenase specific activities of intact forespores could only be explained if 44 and 20% respectively of mother-cell membrane were contaminating this fraction. Even if contamination on this scale were not detectable microscopically, which is unlikely, it is ruled out on the grounds that protein recoveries in these experiments show that at least 90% of mother-cell membrane protein is recovered in the appropriate fraction.

Discussion

In the present study several membrane-bound enzyme activities have been located on the inner surface of the plasma membrane of *B. megaterium*, and this information has been used to show that the outer membrane of the forespore has a reversed orientation relative to the sporangial cell plasma membrane. The enzyme activities studied were ATPase and portions of the electron-transport chain involved with L-malate and NADH.

Considerable evidence (Salton, 1974; van Thienen & Postma, 1973; Futai, 1974; Hare *et al.*, 1974) indicates that ATPases from several different bacteria can be reliably located on the inner surface of the plasma membrane. Examination of plasma-membrane preparations from this organism revealed a $\text{Ca}^{2+}+\text{Mg}^{2+}$ -stimulated ATPase. This activity was undetectable in intact protoplasts but could be easily demonstrated in protoplast lysates, which is consistent with the localization of this activity on the inner surface of the plasma membrane. By contrast intact forespores showed considerable ATPase activity. ATPase activity was recovered virtually entirely in the membrane fractions of protoplasts and forespores.

The L-malate dehydrogenase and NADH dehydrogenase portions of the electron-transport chain were measured spectrophotometrically by following the reduction of 2,6-dichlorophenol-indophenol. Assays of the entire electron-transport chain, i.e. L-malate oxidase and NADH oxidase, were made by using an oxygen electrode. These studies had the advantages of measuring different portions of the electron-transport chain by two different assay methods.

L-Malate dehydrogenase and L-malate oxidase activities could not be detected in intact vegetative and sporangial cell protoplasts, but protoplasts lysed either by sonication or osmotically had appreciable

activity. Essentially all of the L-malate dehydrogenase and L-malate oxidase activities were recovered in the membrane fraction. From these results it seems likely that L-malate dehydrogenase and L-malate oxidase are enzymes of the inner surface of the plasma membrane. This view is supported by the demonstration of a membrane inner-face location of another electron-transport dehydrogenase, DL- α -glycerol phosphate dehydrogenase, by Weiner (1974) in *Escherichia coli*. It is unlikely that *B. megaterium* will be readily permeable to L-malate under our growth conditions, as the transport system for this dicarboxylic acid has been shown to be inducible in *B. subtilis* (Fournier & Pardee, 1974). In contrast with sporulating cell protoplasts, intact stage III and IV forespores had considerable L-malate dehydrogenase and oxidase activities. The activities were recovered in the membrane fraction. *B. megaterium* does possess membrane DL- α -glycerol phosphate oxidase in the exponential phase of growth, but disappearance of this activity during sporulation (Wilkinson & Ellar, 1975) makes this enzyme unsuitable for probing the orientation of forespore membranes.

The absence of detectable NADH dehydrogenase and oxidase activities in intact protoplasts, but their ready detection in stage III and IV forespores, supports the idea that these activities are reliable markers of the inner-membrane surface. NADH oxidase has been located on the inner surface of the plasma membrane in *B. subtilis* (Hampton & Freese, 1974) and *E. coli* (Hare *et al.*, 1974). Intact forespores had a high affinity for NADH oxidation, as would be expected for an outer forespore membrane with a reversed orientation. The low K_m value (about $50\mu\text{M}$) is evidence against the measured NADH oxidase activity of forespores being the result of rate-limited entry of NADH into forespores, such as is the case for intact *B. subtilis* cells, where the K_m for NADH oxidation is 2.1mM (Hampton & Freese, 1974). We did not find any evidence for two K_m values for NADH oxidation by protoplast membrane preparations like those described by Hampton & Freese (1974). It seems possible that our membrane preparations were mostly open or 'inside out', and consequently had high affinity for NADH, since for NADH oxidase assays the protoplasts were lysed at alkaline pH in the absence of Mg^{2+} . These lysis conditions are known to favour formation of 'inside-out' vesicles in erythrocyte preparations (Steck & Kant, 1974). Also, bivalent-cation depletion is known to 'unmask' many membrane enzyme activities (Salton, 1974).

In both dehydrogenase and oxidase assays it was noted that intact protoplasts had greater activities than forespores in the absence of substrates. It could be argued that this higher rate of endogenous activity obscures any stimulation of activity by substrates in protoplasts, and that the increased rate

on substrate addition in forespores is due to the presence of less endogenous activity. This seems unlikely, as exponential-phase and stage II protoplast lysates had two to three times the NADH oxidase activity of intact protoplasts plus NADH (Table 4). Thus it would be expected that if protoplasts were appreciably permeable to NADH or if NADH oxidase was present on the outer surface of the membrane, then a stimulation of activity by NADH would not be obscured. Species of *Bacillus* often accumulate considerable amounts of poly- β -hydroxybutyrate during sporulation, which probably contributes to their endogenous substrates along with the extensive proteolysis occurring in this organism during sporulation (Eaton & Ellar, 1974). Preliminary attempts to deplete endogenous reserves in whole cells, intact protoplasts and forespores have proved difficult.

This criticism of high endogenous enzyme activities is avoided in the ATPase measurements by the nature of the assays, where measurements of P_i present in preparations in the absence of added ATP showed that no increase in P_i content occurred during the incubation and that the content of P_i on a protein basis was essentially the same in intact protoplast and forespore preparations.

Experiments such as these involving substrate accessibility are open to the criticism that detection of various activities in forespores but not in protoplasts is the result of damage to the membranes during forespore preparation. However, the demonstration of comparable swelling and shrinkage of forespore and protoplast suspensions in solutions of non-penetrant solutes of different osmolality strongly support the retention of an intact permeability barrier in the forespores.

Thus our investigations provide biochemical evidence that the outer forespore membrane has a reversed polarity with reference to the sporangial cell plasma membrane, a contention held for some time on the basis of morphological evidence. It seems that this reversed polarity poses unique and interesting problems in the transport of metabolites into the developing spore, and in the site of synthesis of several spore components.

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