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STEADY-STATE FLUORESCENCE ANISOTROPY CHANGES OF 1,6-DIPHENYL-1,3,5,-HEXATRIENE IN MEMBRANES FROM *BACILLUS MEGATERIUM* SPORES

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The steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene incorporated into isolated *Bacillus megaterium* spore membranes was measured. Compounds capable of triggering spore germination in vivo caused an increase in the anisotropy of diphenylhexatriene. These increases in anisotropy of diphenylhexatriene in spore membranes are likely to represent at least a portion of the trigger mechanism for spore germination based on the following observations. First, there was an exceptional positive correlation between compounds that both triggered germination in vivo and caused changes in anisotrpy in vitro. Second. the capacity of membranes to respond to germinants by increases in anisotropy was unique to membranes from spores but disappeared after germination. Third, alteration of spores chemically or genetically to block the in vivo triggering of germination by L-proline also blocked the in vitro anisotropy change with L-proline but not D-glucose. Finally, there was no correlation between the transport activities of specific compounds and the ability of these compounds to either trigger germination or alter the anisotropy of diphenylhexatriene in the membranes. Although we do not known the nature of the molecular interactions giving rise to the anisotropy changes, we hypothesize that they are due to changes in protein conformation that alter protein-protein and/or protein-lipid interactions. Such modifications of membrane structures could account for the rapid release of small molecular weight compounds such as K^+ and Ca^{2+} early in germination.

Introduction

Germination of bacterial spores can be triggered by a few simple compounds, but the mechanism of triggering is unknown. Generally spores are first heat-activated, which has the effect of synchronizing germination and then a trigger compound is added that immediately starts germination. In *Bacillus megaterium* QM B1551, germination can be triggered by a few specific monosaccharides or amino acids, e.g. D-glucose or L-proline, apparently without metabolism [1–3]. We hypothesized that triggering is mediated by components of the spore membrane and the germinant causes a physical change in the membrane, possibly altering its permeability towards water and small molecules [4,5].

Some support for a membane localization of the trigger reaction is based on the following observations. First, an analog of L-proline, proline chloromethyl ketone, will covalently label a membrane protein which was identified as a Coomassie

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Abbreviations: SDS-DTT, sodium dodecyl sulfate-dithiothreitol; Hepes, *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid; PMSF; phenylmethylsulfonyl fluoride.

blue stained band on polyacrylamide gels (Ref. 6; Ugolini and Vary, unpublished data). Second, treatment of spores with acetic anhydride inhibits proline triggering and also labels a similar membrane protein(s) [7]. Third, by electron spin resonance, L-proline caused slight changes in the upper transition temperature of spore membranes in vitro [4]. Less direct evidence has also been reported by nuclear magnetic resonance studies [8].

Shinitzky and co-workers [9,10] described the use of a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene, to detect changes in the lipid environment of membranes. This technique has been commonly used to compare differences in the physical state of membranes of transformed cells [11], activation of blood platelets by thrombin [12], and the effect of oncogenic agents on rat embryo cells [13]. While precise interpretations of anisotropy changes with respect to actual molecular changes in membrane components is somewhat uncertain [14–16], it is still possible to observe measurable events that alter the microenvironment around the probe. In this context we have used steady-state fluorescence anisotropy changes of diphenylhexatriene to study how spore membranes react to trigger and non-trigger compounds in vitro. We have observed a strong positive correlation between the ability of a compound to trigger germination in vivo and to cause diphenylhexatriene anisotropy changes in the spore membrane in vitro. A preliminary report of some of this work has been published [7].

Materials and Methods

Organism, growth conditions and germination. *Bacilluis megaterium* QM B1551 was the wild-type strain and JV-137 was a mutant strain unable to germinate on L-proline [2]. Both strains were grown in a supplemented nutrient broth medium containing D-glucose, salts and nutrient broth at 30°C. Spores were harvested, washed and lyophilized as previously described [3] and stored dry in vacuo. All references to spore weights are on a dry weight basis.

Triggering of spore germination was measured spectrophotometrically as previously described [3] with spores that were heat-activated for 10 min at 60°C.

Membrane isolation. In all cases, spores were first extracted with sodium dodecyl sulfate-dithiothreitol (SDS-DTT) to disrupt the spore coats [17], washed to remove the SDS-DTT, and heat-activated for 10 min at 60°C. Membranes were then isolated by lysis with lysozyme and sonication as previously described [18] except that 2 mM phenylmethylsulfonyl fluoride (PMSF) was added during lysis. These procedures yield mainly spore inner membranes that are biologically active as judged by marker enzyme assays [18] and amino acid transport (this report). All spore membranes were isolated from spores that were heat-activated for 10 min at 60°C although we also isolated membranes from non-heated spores and found results similar to those reported below. The membranes, after purification on sucrose gradients, were suspended in about 1 ml of 10 mM Hepes at pH 7.5, labeled with diphenylhexatriene (to be described below) and centrifuged at 304000 × *g* for 2 h to remove excess diphenylhexatriene. The membrane pellets were resuspended in 10 mM Hepes (pH 7.5) at a protein concentration of 15 to 20 mg/ml, determined by the method of Lowry et al. [19]. Membranes were stored at 3°C and all assays were done within 3 days of membrane isolation.

The same methods as above were used for the isolation of membranes from SDS-DTT extracted spores that had been either germinated or acetylated with acetic anhydride. To isolate membranes from germinated spores, heat-activated spores were resuspended at 50 mg/ml in 0.1 M Hepes (pH 7.5) containing 2 mM PMSF, 200 μg/ml chloramphenicol, and 10 mM D-glucose and incubated for 20 min at 30°C. The spore suspension was cooled on ice, centrifuged at 5000 × *g* for 5 min at 3°C, resuspended and the membranes were isolated as described above. Membranes were also isolated from spores treated with acetic anhydride in vivo which inhibits L-proline-triggered germination [7]. Briefly, heat-activated spores were resuspended at 5 mg/ml in 200 ml of 10 mM sodium borate buffer (pH 9) and acetic anhydride was added to a final concentration of 0.1% (v/v). The suspension was incubated for 10 min at room temperature and the pH was maintained at 9 by addition of 0.1 M NaOH. The acetylated spores were centrifuged at 5000 × *g* for 5 min, washed once with water and membranes

were isolated as described above. Membranes from vegetative cells were isolated from 1 l of cells grown in supplemented nutrient broth at 30°C to an absorbance of 1 at 660 nm. The cells were centrifuged at $5000 \times g$ for 5 min, washed once with a minimal salts medium [20] and the membranes were isolated in the same way as for spore membranes.

Labeling membranes with diphenylhexatriene. To a 15 ml conical test tube, 0.2 ml of 0.2 mM diphenylhexatriene in $CHCl_3$ was added and the $CHCl_3$ was evaporated under N_2. Approximately 1 ml of membrane suspension in 10 mM Hepes (pH 7.5) containing 12 to 15 mg of protein/ml was added to the tube and incubated for 15 min at 25°C in an Ultramet III bath sonicator (Buehler Ltd.) as previously described for labeling membranes with spin-labeled fatty acids [21]. Alternate methods of labeling such as incubating membranes with glass beads coated with diphenylhexatriene or addition of an acetone solution of diphenylhexatriene directly to the membranes [22] were tested, but the method used for these studies gave the most reproducible results. Tetrahydrofuran could not be used as a solvent because it triggered germination [2]. The diphenylhexatriene-labeled membranes were cooled on ice, the volume adjusted to 25 ml with cold 10 mM Hepes (pH 7.5) and centrifuged at $304\,000 \times g$ for 2 h at 3°C to remove any unincorporated probe. The membrane pellet was resuspended in cold 10 mM Hepes, (pH 7.5) at a protein concentration of 15 to 20 mg/ml and stored at 3°C. All of the above steps were carried out with the overhead lights turned off to prevent photoisomerization of diphenylhexatriene [23]. The molar ratio of phospholipid to probe was about 100 : 1. The extent of diphenylhexatriene incorporation into membranes was determined by extracting an aliquot of labeled membranes with $CHCl_3$ and quantitating the fluorescence intensity relative to known concentrations of diphenylhexatriene in $CHCl_3$ (method suggested by Michael Glaser, University of Illinois, Urbana). By this method, > 90% of the diphenylhexatriene was extracted from the membranes and recovered in $CHCl_3$. The phospholipid content was measured as previously described [18].

Fluorescence depolarization studies. Diphenylhexatriene-labeled membranes were diluted with 10 mM Hepes (pH 7.5) to a final protein concentration of 0.5 mg/ml, centrifuged at $5000 \times g$ for 5 min at 3°C to remove particulate matter which could cause light scattering [24], and 2 ml were transferred to 1 cm pathlength cuvettes for fluorescence depolarization studies. Steady-state fluorescence depolarization of diphenylhexatriene was measured with a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with polarization accessories and a thermostatically controled four-cell cuvette holder. Samples were excited at 360 nm and emission was measured at 430 nm with the instrument's 390 nm filter present in the emission beam to minimize contributions from scattered exciting light. Excitation and emission slit widths were set at 6 and 8 nm, respectively. All fluorescence measurements were made in the ratio mode.

Steady-state fluorescence anisotropy (r) is defined as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ represent the intensity of the emission beam polarized parallel and perpendicular to the vertically polarized excitation beam. Anisotropy values were calculated automatically with a Perkin-Elmer DSCU-2 unit according to Weidekamm et al. [25] which includes an emission grating factor (G) correction. Contribution by scattered light to the calculated anisotropy value was determined according to Shinitzky and Barenholz [9] and was less than 2% and was not routinely corrected for [24]. The absorbance of the membrane samples was less than 0.1 and 0.15 at 430 nm and 450 nm, respectively.

The temperature dependence of anisotropy for each sample was determined in the range of 10 to 38°C. Membrane samples were equilibrated to 10°C in a water-jacketed cuvette holder and the temperature was maintained within ± 0.5°C with a Fisher refrigerated bath. The temperature was monitored with a YSI model 42SC Tele-thermometer. Sample temperature was then increased in 2–3°C increments with 12 min of equilibration between each temperature, measurements were made in duplicate or triplicate (differences in values were < 1% of each other) and averaged. After

reaching 38°C, appropriate additions were made to the samples as indicated in the text and the temperature was slowly decreased in 2–3°C increments and anisotropy values measured. This procedure provided an internal consistency for each sample that was very reproducible, as will be shown for Fig. 1 below.

Transport assays. Uptake of radioactive amino acids or glucose by membrane samples was measured using NADH as an energy source by the method of Konings and Freese [26]. Uptake was linear for at least 1 to 2 min and rates were calculated from the linear portions of the curves.

Materials. Chemicals were from Calbiochem or Sigma Chemical Co. Radioactive L-[U-^{14}C]alanine (171 mCi/mmol), L-[U-^{14}C]leucine (348 mCi/mmol), L-[U-^{14}C]isoleucine (342 mCi/mmol), L-[U-^{14}C]glycine (113 mCi/mmol), L-[U-^{14}C]lysine (342 mCi/mmol) and D-[U-^{14}C]glucopyranose (347 mCi/mmol) were from Amersham/Searle. L-[G-^{3}H]Proline was from ICN and 2-deoxy-D-[1-^{14}C]glucose (45 mCi/mmol) was from New England Nuclear.

Results

The steady-state fluorescence anisotropy of diphenylhexatriene was measured in membranes isolated from heat-activated spores. As a control, the temeprature of a labeled membrane preparation was increased from 10 to 38°C and the anisotropy of the incorporated diphenylhexatriene decreased (Fig. 1a). At 38°C, 20 μl of buffer was added to the sample and the temperature was then decreased from 38°C to 10°C. Similar anisotropy values were found as before until around 25°C when the values became consistently lower than those obtained while the temperature was being increased. The same results were found if the experiment was done with no buffer addition at 38°C. These deviations in the anisotropy values below 25°C are assumed to be due to hysteresis as has been observed in studies with membranes utilizing differential scanning calorimetry [24]. When a trigger compound, L-proline, was added to a spore membrane preparation at 38°C, there was an increase in anisotropy (Fig. 1b). As the temperature was lowered, the anisotropy values were consistently higher than those obtained before L-proline addition and below 25°C, the hysteresis seen in the control was no longer observed. We also tested D-proline, which does not trigger germination [2], and it did not cause an increase in anisotropy (Fig. 1c). Below 25°C, the values for anisotropy were slightly lower after D-proline addition than before, an effect that was similar but not identical to the hysteresis observed with the control containing no proline (Fig. 1a).

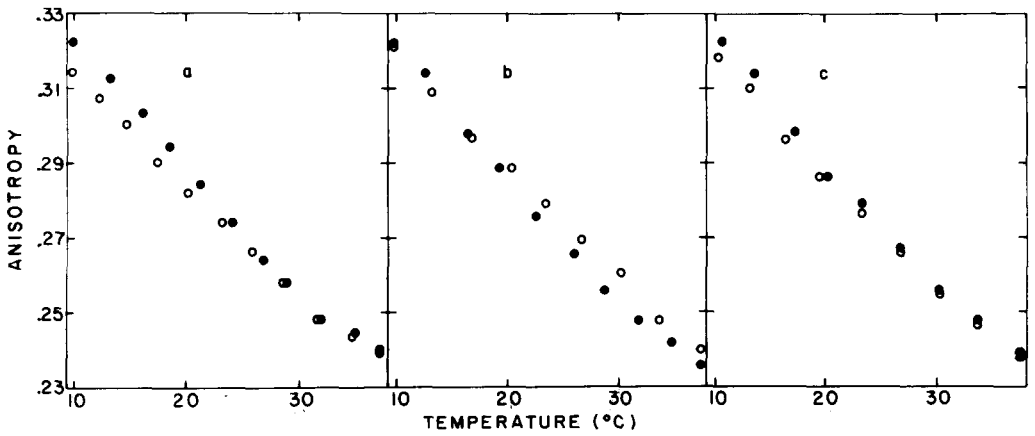

Fig. 1. Temperature dependence of the steady-state fluorescence anisotropy of diphenylhexatriene incorporated into isolated spore membranes. The anisotropy of diphenylhexatriene incorporated in membranes from heat-activated spores (0.5 mg of protein/ml of 10 mM Hepes buffer, pH 7.5) was determined as a function of temperature as described in the text. The temperature was first increased from 10 to 38°C (●), then decreased (○) after the addition of: (a) 20 μl of 10 mM Hepes (pH 7.5), (b) 20μl of 1 M L-proline or (c) 20 μl of 1 M D-proline. The final concentration of proline was 10 mM.

TABLE I

EFFECT OF AMINO ACIDS AND MONOSACCHARIDES ON ANISOTROPY, GERMINATION AND TRANSPORT

The indicated compounds were added to a final concentration of 10 mM exactly as described in the legend of Fig. 1. The anisotropy of DPH incorporated into membranes from heat-activated spores was measured as described in the text. The average changes in anisotropy ($r \pm$ S.D.) at 30.5°C were calculated from n experiments done as in Fig. 1 and the values were multiplied by 1000 for convenience in constructing the table. Where only a few experiments were done, the individual changes in r are listed for each experiment separated by commas. Germination triggered by the indicated compounds (10 mM final concentration) is indicated from previous results for some amino acids [17] and monosaccharides [3,28] and also confirmed by us for each compound with spores from the same batch used to isolate membranes for anisotropy measurements. Transport was measured as described in the text and is expressed as nmol/min per mg of protein.

Compounds	Change in anisotropy $r \pm$ S.D. (n) ($\times 10^3$)	Germination	Transport
Buffer	0 ± 1 (5)	−	
L-Proline	8 ± 3 (26)	+	700
D-Proline	0 ± 3 (16)	−	
L-Leucine	7, 7 (2)	+	140
D-Leucine	0 (1)	−	
L-Arginine	0 (1)	−	
L-Lysine	0 (1)	−	100
L-Alanine	1, −2 (2)	−	1100
L-Serine	−1 (1)	−	
L-Glycine	−1 (1)	−	600
L-Valine	−1 (1)	−	
L-Isoleucine	1 (1)	−	80
D-Glucose	7 ± 3 (11)	+	0
L-Glucose	1 ± 2 (6)	−	
Methyl α-D-glucoside	7, 6 (2)	+	
D-Fructose	8, 8 (2)	+	
2-Deoxy-D-glucose	8 (1)	+	0
D-Talose	1 (1)	−	
D-Ribose	1 (1)	−	

From several experiments done exactly as shown in Fig. 1, the changes in anisotropy ($\times 10^3$) at 30.5°C were averaged and the standard deviations were calculated (Table I). In the buffer control, the average change in anisotropy ($\times 10^3$) was 0 ± 1 with 95% confidence limits from −1 to 2. However with L-proline the average change in anisotropy ($\times 10^3$) was 8 ± 3 with 95% confidence limits from 7 to 9. With D-proline, the average change in anisotropy ($\times 10^3$) was 0 ± 3 with 95% confidence limits from −2 to 1. The increases in anisotropy values caused by L-proline are small, but in the same range as reported polarization values for the effect of insulin and insulin analogs on liver plasma membranes [27]. In that study, we calculate that insulin caused an average increase in polarization values ($\times 10^3$) of 13 with a standard deviation of 6 at 37°C (Table I, Ref. 27).

To determine if there was a correlation between the ability of a compound to trigger germination in vivo and cause a change in anisotropy in vitro, several other amino acids and some monosaccharides were examined. L-Leucine but not D-leucine caused an increase in anisotropy and triggered germination. No other amino acid had this capacity. With monosaccharides, those that triggered germination, e.g. D-glucose or 2-deoxy-D-glucose, caused an increase in anisotropy similar to L-proline. Other monosaccharides that did not trigger germination, e.g. L-glucose, D-talose or D-ribose, had very small effects on the anisotropy of diphenylhexatriene. We have not included all compounds known to be germinants. For instance, we have not included halide salts because they quench

diphenylhexatriene fluorescence and the addition of calcium salts causes precipitation of membrane phospholipids. In all other cases, we have found no exception to the correlation between triggering in vivo and changes in anisotropy in vitro that are caused by specific germinants.

The data in our experiments show that L-proline and other trigger compounds specifically alter spore membranes in vitro as monitored by changes in the anisotropy of diphenylhexatriene. Similar results were found with membranes from spores with no heat-activation. Also, the reported increases in anisotropy with L-proline were a property of the intact membrane, since the isolated phospholipids did not respond to L-proline (data not shown).

It is possible that the changes in anisotropy reported here might reflect the transport of the tested compounds. If so, compounds that are transported by these membranes might also cause increases in anisotropy. This was tested by measuring the uptake of several amino acids with the same membrane preparation (containing diphenylhexatriene) that was used to measure anisotropy. In each case, amino acid uptake was linear for 1 to 2 min and had an absolute requirement for reduced NADH. As shown in Table I, amino acids were transported with rates varying from 80 to 1100 nmol/min per mg of protein and there was no correlation between the ability to trigger germination (or change anisotropy) and the ability to be transported by membranes. The differences in rates for the different amino acids are similar to those found with *B. subtilis* vegetative cell membranes [26]. With monosaccharides, there was no uptake of radioactive D-glucose or 2-deoxy-D-glucose which might be expected since spores do not contain a detectable phosphotransferase system [1]. However, both sugars could trigger germination. Therefore, it appears unlikely that the transport systems for various germinants could alone account for the observed changes in diphenylhexatriene anisotropy, although we have not ruled out that one or more components of different transport systems could be involved.

If the observed changes in anisotropy of diphenylhexatriene in spore membranes reflects an aspect of the germination trigger reaction, one would not expect to observe similiar changes with membranes from vegative cells since triggering of germination is presumably spore specific. Therefore, membranes were isolated from vegetative cells, labeled with diphenylhexatriene and anisotropy values were measured as a function of temperature. When L-proline was added there were no increases in anisotropy (Fig. 2a) as observed for membranes from heat-activated spores. Similarly, L-leucine and D-glucose did not increase the anisotropy of diphenylhexatriene in vegetative cell membranes indicating that the capacity of membranes to react specifically with germinants is not present in vegetative cells. It may be noted that the steady-state fluorescence anisotropy values for membranes isolated from vegetative cells were lower than those from heat-activated spores (note scale in Fig. 2a). Whether this difference was due to an increased 'fluidity' of vegetative cell membrane lipids is unknown, since all of the different factors that contribute to an anisotropy value were not determined. However, the lower anisotropy values would tend to agree with fatty acid analyses which show that these membranes contain a higher proportion of lower melting point fatty acids than spore membranes [29].

We have also isolated membranes from spores that were first allowed to trigger germination on D-glucose for 30 min. As shown in Fig. 2b, the anisotropy values were slightly less than observed with membranes from heat-activated spores, but

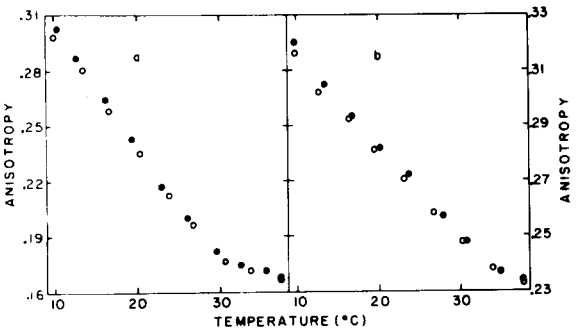

Fig. 2. Membranes from vegetative cells and germinated spores. The anisotropy of diphenylhexatriene incorporated into membranes isolated from vegetative cells (a) and germinated spores (b) was determined as in the legend to Fig. 1. The temperature was first increased (●), and then decreased (○) following the addition of 20 μl of 1 M L-proline (final concentration 10 mM). Each experiment was done twice and the changes in anisotropy at 30.5°C were zero in each case.

more important, L-proline had no effect on these anisotropy values. The same negative results were obtained with L-leucine or D-glucose. Therefore, it appears that the germinant-induced changes in anisotropy of diphenylhexatriene is specific to membranes from spores only before germination.

To examine further the relationship between changes in anisotropy and the triggering of germination in vivo, membranes were isolated from heat-activated spores that had been treated with acetic anhydride prior to membrane isolation. Previous studies showed that triggering of germination by L-proline but not D-glucose was inhibited with acetic anhydride treatment of spores [7]. Therefore, if the changes in anisotropy reflect some aspect of the trigger reaction, membranes from acetylated spores should not respond to L-proline but should respond to D-glucose. Membranes were isolated from spores that had been acetylated with acetic anhydride and L-proline caused very little change in anisotropy as reported by diphenylhexatriene (Table II). When D-glucose was added to these membranes, an increase in anisotropy comparable to that seen with membranes from non-acetylated spores was observed. As controls, D-proline or L-glucose caused little change in anisotropy of diphenylhexatriene. These results further support the correlation between changes in anisotropy and the triggering of spore germination.

Finally, mutant JV-137, which cannot trigger germination on L-proline but can on D-glucose, was examined. When L-proline was added to membranes isolated from JV-137 spores there was no change in anisotropy (Table II). However, in the presence of D-glucose, the increase in anisotropy was similar to that observed with membranes from wild-type spores. Again, D-proline or L-glucose caused little change in anisotropy of diphenylhexatriene in spore membranes.

TABLE II

MEMBRANE ANISOTROPY CHANGES WITH PROLINE OR GLUCOSE

Membranes were isolated from wild-type spores that had been treated with acetic anhydride or from mutant JV-137 spores as described in the text. All other conditions are the same as Table I.

Membranes	Changes in anisotropy $r \pm$S.D. (n) ($\times 10^3$)	Germination
Membrane from acetic anhydride-treated spores		
+ L-Proline	1 ± 2 (6)	−
+ D-Proline	0, 0 (2)	−
+ D-Glucose	7 ± 1 (5)	+
+ L-Glucose	1 ± 1 (3)	−
Membranes from mutant JV-137 spores		
+ L-Proline	0, 0 (2)	−
+ D-Proline	−1 (1)	−
+ D-Glucose	8 ± 2 (3)	+
+ L-Glucose	1, 0 (2)	−

Discussion

This study was aimed at investigating a possible relationship between the spore membrane and the triggering of germination. We have measured the steady-state fluorescence anisotropy of diphenylhexatriene incorporated into spore membranes to monitor changes in the physical properties of the membranes. Although previous studies [27,30] have related changes in the anisotropy of diphenylhexatriene to alterations in the fluidity of the membrane lipids, more recent works [23,31] have shown that restrictions imposed on the rotational mobility of diphenylhexatriene reflected by anisotropy are not solely due to the viscous drag in the probe's environment. The anisotropy values also contain contributions due to the structural hinderance of the probe's rotation and the anisotropic motion of the probe. Therefore, the changes in anisotropy presented in this study represent qualitative changes as done in other studies [12,27] without distinguishing between different mechanisms that might affect the rotational freedom and the fluorescent lifetime of the probe. For instance, pertubation of the membrane structure due to alteration of protein-protein or protein-lipid interactions could account for changes in the probe's environment. Protein-protein interactions, such as clustering, could give rise to decreased rotational mobility of the probe while alterations in protein conformation could cause a vertical displacement of proteins into the membrane interior thus altering the packing density of the membrane [27].

By measuring anisotropy of diphenylhexatriene incorporated into spore membranes, L-proline but not D-proline caused an increase in anisotropy. While these studies do not show the physical basis for the induced change in anisotropy, the observed increase in anisotropy may reflect the activity of portions of the trigger mechanism. We tested the commonly known amino acid and monosaccharide germinants and found a striking correlation between the capacity to trigger germination in vivo and to change the anisotropy of diphenylhexatriene in vitro. We also tested several compounds known not to be germinants and none changed the diphenylhexatriene anisotropy in spore membranes. We did not make a complete survey of all amino acids and monosaccharides but only those known to trigger germination and a selection of those that did not. There is only one possible exception in that L-alanine has been shown by others to trigger germination in this strain [32], but in our hands with freshly grown and washed spores, L-alanine did not trigger germination [17].

Membranes isolated from vegetative cells or germinated spores did not have the capacity to respond to germinants as measured by increased anisotropy of diphenylhexatriene. Therefore the germinant-induced change in anisotropy must be spore specific. While this result was expected for membranes from vegetative cells it was not apparent a priori for membranes from germinated spores and suggests a spore membrane property or component(s) that is synthesized during sporulation, present in spores but inactivated or degraded after germination. The possibility of measuring this sequence of events is currently under investigation. Most workers in the field recognize that spores may go through some germination-like reactions during disruption. Therefore, it is significant that only membranes from heat-activated spores and not germinated spores exhibited the increased anisotropy of diphenylhexatriene with germinants, yet both types of spore were disrupted identically. While we may not have succeeded in isolating the intact trigger mechanism in the membrane, at least a portion of a spore-specific reaction was isolated that reacts with germinants specifically. Also one might presume that the physical properties of the spore inner membranes may change dramatically during germination and we might be able to observe only a portion of the in vivo changes by our in vitro tests. We cannot directly assess the above differences by our methods but such a comparison may be possible by our nuclear magnetic resonance experiments which are in progress.

There are two ways that we can specifically block L-proline triggered germination by either acetylation of spores with acetic anhydride or with spores from a mutant, JV-137. Membranes isolated from spores of either the above sources still responded to D-glucose addition by showing increased anisotropy of diphenylhexatriene, but L-proline was ineffective. The site of action of L-proline in spore membranes was apparently blocked by prior acetic anhydride treatment or lacking in the mutant spores. We are currently attempting to identify this site which is presumably a membrane protein as suggested by preliminary experiments [6,7].

During bacterial spore germination, one of the earliest events detected has been a change in the permeability of the spore membrane (presumably the spore inner membrane) towards small molecules, resulting in the efflux of Na^+, K^+, Ca^{2+} and dipicolinic acid [33]. Therefore trigger compounds could cause changes in protein conformation in the membrane that might facilitate the exchange of small molecules across the membranes as one of the earliest reactions in germination. Studies in other systems have suggested a role of proteins in mediating the response of cells to external chemical stimuli such as chemotaxis [34], lectin-induced capping of membrane proteins [35], and hormonal regulation of the biological activity of cells [36]. Luly and Shinitzky [27] reported that binding of insulin to liver cells increased the microviscosity of those membranes. Other studies on the effect of thrombin on platelet cells [12] and glucose on pancreatic cells [37] have reported changes in membrane fluidity induced by the interaction with specific chemical stimuli. We have now shown that a very different and unique chemical signal for triggering germination may also be a membrane-mediated response.

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References

1 Racine, F.M., Dills, S.S. and Vary, J.C. (1979) J. Bacteriol. 138, 442–445
2 Rossignol, D.P. and Vary, J.C. (1979) J. Bacteriol. 138, 431–441
3 Shay, L.K. and Vary, J.C. (1978) Biochim. Biophys. Acta 538, 284–292
4 Janoff, A.S., Coughlin, R.T., Racine, F.M., McGroarty, E.J. and Vary, J.C. (1979) Biochem. Biophys. Res. Commun. 89, 565–570
5 Vary, J.C. (1978) In Spores VII, (Chambliss, G. and Vary, J.C., eds.), pp. 104–108, American Society for Microbiology, Washington, DC
6 Rossignol. D.P. and Vary, J.C. (1979) Biochem. Biophys. Res. Commun. 89, 547–551
7 Racine, F.M., Skomurski, J.F. and Vary, J.C. (1981) in Sporulation and Germination, (Levinson, H.S., Sonenshein, A.L. and Tipper, D.J. eds.), pp. 224–227, American Society for Microbiology, Washington, DC
8 Stewart, G.S.A.B., Eaton, M.W., Johnstone, K., Barrett, M.D. and Ellar, D.J. (1980) Biochim. Biophys. Acta 600, 270–290
9 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
10 Shinitsky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–149
11 Pessin, J.E., Salter, D.W. and Glaser, M. (1978) Biochemistry 17, 1997–2004
12 Nathan, I., Fleischer, G., Livne, A., Dvilansky, A. and Parola, A.H. (1979) J. Biol. Chem. 254, 9822–9828
13 Fisher, P.B., Flamm, M., Schachter, D. and Weinstein, I.B. (1979) Biochem. Biophys. Res. Commun. 86, 1063–1068
14 Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1980) J. Biol. Chem. 252, 2163–2169
15 Dale, R.E., Chen, L.A. and Brand, L. (1977) J. Biol. Chem. 252, 7500–7510
16 Lackowicz, J.R. (1980) J. Biochem. Biophys. Methods. 2, 91–119
17 Vary, J.C. (1973) J. Bacteriol. 116, 797–802
18 Racine, F.M., and Vary, J.C. (1980) J. Bacteriol. 143, 1208–1214
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Lammi, C.J. and Vary, J.C. (1972) in Spores V (Halvorson, H.O., Hanson, R. and Campbell, L.L., eds.). pp. 277–283, American Society for Microbiology, Washington, DC
21 Janoff, A.S., Haug, A. and McGroarty, E.J. (1979) Biochim. Biophys. Acta 555, 56–66
22 Shinitzky, M., Dianoux, A.C., Gitler, C. amd Weber, G. (1971) Biochemistry 10, 2106–2113
23 Mely-Goubert, B. and Freedman, M.H. (1980) Biochim. Biophys. Acta 601, 315–327
24 Brasitus, T.A., Tall, A.R. and Schachter, D. (1980) Biochemistry 19, 1256–1261
25 Weidekamm, E., Schudt, C. and Brdiczka, D. (1976) Biochim. Biophys. Acta 443, 169–180
26 Konings, W.N. and Freese, E. (1972) J. Biol. Chem. 247, 2408–2418
27 Luly, P. and Shinitzky, M. (1979) Biochemistry 18, 445–450
28 Hyatt, M.T. and Levinson, H.S. (1964) J. Bacteriol. 91, 1811–1818
29 Skomurski, J.F. and Vary, J.C. (1982) Lipids 17, 914–923
30 Stubbs, G.W., Litman, B.J. and Barenholz, Y. (1976) Biochemistry 15, 2766–2772
31 Jahnig, F. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6361–6365
32 Levinson, H.S. and Hyatt, M.T. (1962) J. Bacteriol. 83, 1224–1230
33 Swerdlow, B.M., Setlow, B. and Setlow, P. (1981) J. Bacteriol. 148, 20–29
34 Springer, M.S., Goy, M.F. and Adler, J. (1979) Nature 280, 279–284
35 Nicolson, G.L. (1976) Biochim. Biophys. Acta 458, 1–72
36 Czech, M.P. (1977) Annu. Res. Biochem. 46, 359–384
37 Deleers, M., Ruysschaert, J.-M. and Malaisse, W.J. (1977) Biochem. Biophys. Res. Commun. 98, 255–260