The Role of Surface Charge in Ionic Germination of *Clostridium perfringens* Spores

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The surface charge against pH behaviour of spores of *Clostridium perfringens* type A was determined using a colloid titration method in the pH range 4–9. The native spores as well as various ionic forms of the spores loaded with divalent cations were found to be negatively charged. A positive colloid, MGCh, inhibited the ionic germination of the spores and this inhibition was highly dependent on pH. However, another positive colloid, GCh, did not inhibit the germination. Chemical modification of carboxyl groups on the spore surface by use of a water-soluble carbodiimide with two nucleophiles, glycine ethyl ester and taurine, caused spores not only to have little or no colloidal charge but also to lose the ability to germinate. The modification of carboxyl groups in the spore coat protein also resulted in elimination of, or marked reduction in its negative charge. These results suggest that carboxyl groups in the spore coat protein are the major negatively charged species on the spore surface. The role of surface charge in the ionic germination of the spores is discussed.

INTRODUCTION

Since the early work of Douglas (1955), which showed that bacterial spores were negatively charged, a few reports concerning microelectrophoretic mobility of spores have appeared in which it has been suggested that carboxyl groups are responsible for the negatively charged species on the spore surfaces (Tochikubo *et al.*, 1975; Gore *et al.*, 1978; Wyatt *et al.*, 1982). However, little is known about the function of the charged species present in the spore surface, in particular the possible involvement of the charged carboxyl groups in germination. Nishihara *et al.* (1981), using the colloid titration method (Watanabe & Takesue, 1976), measured the surface charge of *Bacillus megaterium* QM B1551 spores and concluded that there existed two types of the negatively charged species on the spore surface, one being strong acidic groups such as phosphates and the other being weak acidic groups such as carboxylates. They also suggested that the phosphate residues might block the action of ionic germination and the carboxylate residues might be involved in the later stage of germination. However, their data gave no evidence that phosphate or carboxylate residues present in the spore coat are attributable to the major sites of the negatively charged species on the spore surface.

In this paper, we attempt to locate the sites of carboxyl charges on the spore surface of *Clostridium perfringens* by comparing the colloid titration values of native spores with those in which the carboxyl charges have been chemically modified or eliminated. We also describe an important role of the charged carboxyl groups arising from the spore coat protein in ionic germination of the spores.

METHODS

Organism and preparation of spores. *Clostridium perfringens* type A strain, National Collection of Type Cultures 8238 (Hobbs' serotype 2) was used. The cultures were maintained in cooked meat medium (Difco). Spores were

Abbreviations: GCh, glycol chitosan; MGCh, methylglycol chitosan.

prepared by the method of Duncan & Strong (1968) and were freed of vegetative cells by mild ultra-sonication followed by extensive washings with deionized water. They were stored in water at 2 °C.

Preparation of ionic forms of spores. Aqueous suspensions of the spores were converted to the hydrogen form (H-spores) by titration to pH 4 with 0.01 M-HNO₃ (Alderton & Snell, 1963). Spores loaded with Ca²⁺, Mg²⁺, Sr²⁺, and Ba²⁺ were prepared by titrating aqueous suspensions of H-spores to pH 9 with the hydroxide of these metals (Levinson & Hyatt, 1969).

Germination of spores. Heat-activated (80 °C for 10 min) spores were germinated in a KCl medium consisting of 50 mM-KCl and 50 mM-potassium phosphate buffer (pH 7.0) at 40 °C. The optical density changes were measured using a Klett-Summerson photoelectric colorimeter (filter no. 56). Germination was also confirmed by phase-contrast microscopy.

Colloid titration. The slightly modified method of Watanabe & Takesue (1976) was used. Instead of protamine sulphate, GCh and MGCh (Wako Pure Chemical Corp.) were used as positive colloids, since Nishihara *et al.* (1981) reported that titration values were estimated to be higher with protamine sulphate than with MGCh. An excess amount of the colloid (0.005 M, 5 ml) was added to an appropriate concentration of spores (20–30 mg per 2 ml) and the mixture was adjusted to the required pH with 0.05 M-HCl or 0.05 M-NaOH. After incubation at room temperature for 20 min, the uncombined colloid was back-titrated with 0.0025 M-potassium polyvinyl sulphate (Wako Pure Chemical Corp.) using toluidine blue as the indicator. A sharp end-point with MGCh was obtained by removing the spores by centrifuging the titration mixture and adjusting the pH of the supernatant to 2.5 with 0.05 M-HCl before titration, according to the method of Nishihara *et al.* (1981).

Preparation of spore integuments and coat protein. Spores were disrupted by shaking with glass beads in a Braun cell homogenizer as described previously (Ando, 1979). After removal of the glass beads, the disrupted spore suspension was centrifuged at 12000 g for 20 min. The pellet was washed several times with deionized water at 4 °C. Spore coat protein was extracted from the spore integuments as follows. Spore integuments (6.2 mg ml⁻¹) were treated with lysozyme (100 µg ml⁻¹) in 0.01 M-Tris/HCl buffer, pH 7.8, at 30 °C for 60 min before washing several times with deionized water. After lysozyme treatment, the coat fraction (5 mg ml⁻¹) was suspended in a freshly prepared solution of 50 mM-DTT. The pH was adjusted to 10.5 with 0.1 M-NaOH. The suspension was incubated at 40 °C for 60 min, and centrifuged. The resulting supernatant containing coat protein was dialysed against deionized water and lyophilized.

Chemical modification of carboxyl groups. Two nucleophiles of different electrical charge were coupled with carboxyl groups by the carbodiimide reaction (Hoare & Koshland, 1967). One was glycine ethyl ester which neutralized the carboxyl charges and the other was taurine which, after coupling, remained negatively charged. Spores (10 mg ml⁻¹) were suspended in 10 ml aqueous 0.2 M-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Wako Pure Chemical Corp.) with 0.5 M-nucleophile. The mixture was continuously stirred for 6 h at 25 °C. The pH was adjusted to 4.75 and this value was maintained by addition of 0.05 M-NaOH. The treated spores were washed several times with deionized water by centrifugation. Spore coat protein was treated essentially as described above.

To detect modified carboxyl groups, 3 mg of each sample was hydrolysed in 6 M-HCl at 110 °C for 20 h in a sealed Pyrex-glass tube, and the amounts of glycine and taurine, as compared to untreated control, were determined by a Hitachi model 835 Amino Acid Analyser.

RESULTS

Surface charge of spores against pH behaviour

Figure 1 shows the pH dependence of the colloidal charge of *Cl. perfringens* spores titrated with MGCh or GCh. The colloidal charge was almost zero at pH 4 but increased steadily toward negative values as the pH was raised from 4 to 9 when the spores were titrated with MGCh. Little or no charge was observed between these pH values when the spores were titrated with GCh. This implies that weak acidic groups such as carboxyl groups are the only charged species on the spore surface because MGCh reacts with all negatively charged groups including carboxyl groups, while GCh dissociates at pH < 6.5 and reacts only with strong acidic groups dissociable even at low pH (Nishihara *et al.*, 1981).

Surface charge and germination ability of various ionic forms of spores

Table 1 shows variations in colloidal charge at three different pHs for various ionic forms of the spores, along with the ability of these spores to germinate in the KCl medium. The colloidal charges of all these ionic forms of the spores were zero at pH 4 but increased to more negative values with increasing pH values, as in the case of native spores. However, the colloidal titration values of these ionic forms of the spores were about half those of native or H-spores. The

Fig. 1

Fig. 1. pH-colloidal charge curve of *Cl. perfringens* spores. Spores were titrated with MGCh (○) or GCh (●).

Fig. 2

Fig. 2. Effect of pH on the inhibition of the ionic germination of *Cl. perfringens* spores by MGCh. The heat-activated spores were preincubated in 50 mM-Tris/HCl buffer alone (open symbols), or containing MGCh (closed symbols) at varying pHs for 20 min at 40 °C, and then KCl was added to make its concentration 50 mM at the zero time. ○, ●, pH 7.5; △, ▲, pH 8.0; □, ■, pH 8.5.

Table 1. Colloidal charge and germination ability of various ionic forms of *Cl. perfringens* spores

Values for colloidal charge are given as $\mu\text{equiv. (mg spore dry wt)}^{-1}$ and for germination ability as percentage fall in OD after 60 min.

Ionic form	Colloidal charge			Germination ability
	pH 4	pH 7	pH 9	
Native	0	-0.18	-0.38	64
H ⁺	0	-0.21	-0.39	15
Ca ²⁺	0	-0.13	-0.20	56
Sr ²⁺	0	-0.12	-0.23	62
Ba ²⁺	0	-0.11	-0.21	55
Mg ²⁺	0	-0.13	-0.23	64

germination ability of these spores except H-spores was almost the same. The rate of germination of H-spores was very slow in the KCl medium, but they finally germinated to the same extent that native spores normally attained within 60 min.

Effect of MGCh on germination

Since it was possible that MGCh reacted with some carboxyl groups on the spore surface to neutralize their negative charge, the effect of MGCh on the ionic germination of the spores was studied. Heat-activated spores were preincubated at 40 °C with MGCh [$10 \mu\text{equiv. (mg spore)}^{-1}$] in 50 mM-Tris/HCl buffers of varying pH values for 20 min, and then KCl was added to the mixture to give a final concentration of 50 mM. In the absence of MGCh, changes in pH values between 7.5 and 8.5 exerted little measurable effect on the ionic germination of the spores (Fig. 2). However, germination was strongly inhibited in the presence of MGCh and this inhibition was highly pH-dependent (Fig. 2). Although the inhibitory action of MGCh was most effective at pH 8.5, there was a complete reversal of the inhibition when the inhibitor was removed by washing with water. When MGCh was replaced by GCh, no inhibition of germination was observed over the pH range 6.5 to 7.5.

Table 2. *Amino acid composition of integuments from chemically modified spores*

Values are given as molar percentages.

Amino acid	Glycine-ethyl-ester-modified spores	Taurine-modified spores
Taurine	0.0	6.8
Aspartic acid	10.0	10.4
Threonine	4.0	4.2
Serine	5.4	6.3
Glutamic acid	13.1	13.3
Proline	3.8	4.4
Glycine	16.7	7.6
Alanine	9.7	10.2
Valine	4.4	3.8
Methionine	1.4	1.3
Isoleucine	4.3	3.9
Diaminopimelic acid	2.5	3.1
Leucine	6.0	5.9
Tyrosine	2.8	3.2
Phenylalanine	3.1	3.0
Lysine	7.2	6.7
Histidine	1.8	1.8
Arginine	4.2	4.3

Table 3. *A comparison in colloidal charge and germination ability between modified and unmodified spores*

Values for colloidal charge are the same as those in the legend to Table 1 and those for germination ability are given as percentages of phase-dark spores after 60 min. The following media were used for germination at the indicated pHs. 50 mM-KCl + 50 mM-sodium acetate buffer (pH 4); 50 mM-KCl + 50 mM-sodium phosphate buffer (pH 7); 50 mM-KCl + 50 mM-Tris/HCl buffer (pH 9).

Modified spores	Colloidal charge			Germination ability		
	pH 4	pH 7	pH 9	pH 4	pH 7	pH 9
Glycine-ethyl-ester-modified spores	0	0	0	0	0	0
Taurine modified spores	0	-0.03	-0.06	0	0	0
Unmodified spores	0	-0.18	-0.38	6	97	81

Modification of carboxyl groups on the spore surface

The native spores were treated as described in the text with water-soluble carbodiimide in the presence of either glycine ethyl ester or taurine. Amino acid analyses of the integuments from glycine-ethyl-ester-modified spores (GE-spores) and taurine-modified spores (T-spores) were compared with one another (Table 2). Taurine was detected in large quantities in T-spores but none of it in GE-spores. On the contrary, the glycine content of GE-spores was about twice as much as that of T-spores. These results indicated that approximately equal numbers of carboxyl groups on the surface were affected by the modification with glycine ethyl ester or taurine. The modification of carboxyl groups yielded spores with little or no colloidal charges at the three pHs, as compared to unmodified spores (Table 3). T-spores had slightly negative values at pH 7 and 9, while GE-spores had a zero colloid titration value irrespective of pH. Furthermore, the modification of carboxyl groups resulted in complete loss of ability to germinate even at pH 7, where optimum germination of unmodified spores was observed (Table 3).

Modification of carboxyl groups in the spore coat protein

To determine if most of the carboxyl groups on the spore surface are located in the spore coat, coat protein was extracted with DTT at pH 10.5, and its colloidal charges before and after modification of its carboxyl groups were compared. Since it was impossible to extract coat

Table 4. *Amino acid composition of modified and unmodified spore coat proteins*

Values are given as molar percentages.

Amino acid	Glycine-ethyl- ester-modified coat protein	Taurine- modified coat protein	Unmodified coat protein
Taurine	0.0	8.1	0.0
Aspartic acid	11.8	12.4	13.5
Threonine	3.3	3.5	3.7
Serine	4.3	4.2	4.6
Glutamic acid	11.9	12.3	13.5
Proline	4.6	3.8	4.1
Glycine	18.7	9.6	10.3
Alanine	4.0	4.1	4.4
Valine	4.6	4.6	4.9
Methionine	1.5	1.5	1.7
Isoleucine	4.8	4.8	5.1
Leucine	7.5	7.6	8.2
Tyrosine	3.7	3.7	4.6
Phenylalanine	3.7	3.7	4.0
Lysine	8.2	8.5	9.2
Histidine	2.4	2.4	2.6
Arginine	5.1	5.2	5.5

Table 5. *Colloidal charge of modified and unmodified spore coat proteins*Values are given as $\mu\text{equiv. (mg dry wt protein)}^{-1}$. The buffers used are given in Table 3.

Coat protein	Colloidal charge		
	pH 4	pH 7	pH 9
Glycine-ethyl-ester- modified coat protein	+0.02	+0.02	+0.05
Taurine-modified coat protein	0	-0.08	-0.33
Unmodified coat protein	0	-0.54	-0.82

protein from the integuments of the chemically modified spores due to difficulties in solubilizing it into the alkaline DTT, spore coat protein extracted from native spores was treated for the modification of carboxyl groups. Table 4 shows a comparison of amino acid composition of the coat protein before and after modification of carboxyl groups with either glycine ethyl ester or taurine. These results showed that the coat protein was characterized by a relatively high content of acidic amino acids (aspartic and glutamic). The numbers of taurine and glycine residues incorporated were almost the same and corresponded to more than one half of that of either aspartic or glutamic residues. The colloid titration of native coat protein showed that the net charge was zero at pH 4 but increased toward more negative values with increasing pH values (Table 5). However, the modification of carboxyl groups in the coat protein resulted in the elimination of, or marked reduction in its negative charge. Glycine-ethyl-ester-modified coat protein had slightly positive charges irrespective of pH, while taurine-modified coat protein had much less negative charges dependent on pH than those of unmodified coat protein.

DISCUSSION

The surface charge of *Cl. perfringens* spores was found to be negative at $\text{pH} > 4$ from the pH-colloidal charge curve (Fig. 1), suggesting that the only ionizable species on the spore surface are weakly acidic groups such as carboxyl groups. These results are in agreement with those from

microelectrophoretic studies on some *Bacillus* spores. However, a difference in surface charges of spores in terms of the colloid titration was observed between *Cl. perfringens* and *Bacillus megaterium*. The negatively charged groups of the latter spores comprised mainly phosphates and carboxylates because they were titratable with both GCh and MGCh. Since measurement of colloid titration value provides information only about the surface of a spore as a colloid particle, the difference may be due to some morphological and chemical properties of the spore surfaces, in particular of the outermost layers of the spore coats. The outer coat of *B. megaterium* spores was constituted mainly by a resistant fraction containing phosphogalactosamine (Kondo & Nishihara, 1970), whereas the outer coat of *Cl. perfringens* spores was composed mainly of DTT-alkali-soluble protein (Labbe *et al.*, 1978). Therefore, colloid titration values of these spores are concerned with the degree of dissociation of these components in the outer coats, which should be influenced by the pH or ionic strength of the surroundings.

The ionic germination of *Cl. perfringens* spores was greatly inhibited in the presence of MGCh and the inhibition was highly dependent on pH, suggesting that MGCh-reactive carboxyl groups on the spore surface may play an important role in the ionic germination of the spores. MGCh might combine reversibly with the carboxyl groups by neutralizing their negative charge, thus leading to the inhibition of certain events early in germination. The inhibitory action of MGCh was complete as determined by the microscopic observation. This was in contrast to the case of *B. megaterium* spores, in which germination was partially inhibited to yield semi-refractile spores. Nishihara *et al.* (1981) suggested that the action of MGCh on germination might be the fixation of the coat components by reaction with the carboxyl groups, leading to the prevention of the second stage of the germination. These discrepancies may also be accounted for by the difference in the morphological and chemical properties between the surfaces of these spores.

The presence of carboxyl groups on the spore surface was further evidenced by investigating the effect of water-soluble carbodiimide, a reagent for modifying free carboxyl groups, on the colloidal charge of the spores. Glycine-ethyl-ester-modified spores had zero colloidal charge irrespective of pH, indicating a direct result of the elimination of the negative charge due to the carboxyl groups. In addition, these modified spores could no longer germinate in the KCl medium. This also means that some negatively charged carboxyl groups are involved in the ionic germination of the spores. The chemical modification of the carboxyl groups in the spore coat protein caused a significant reduction in colloidal charge, suggesting that the negatively charged loci of the spore surface are acidic proteins in the spore coat.

Although a number of studies have been made on the chemical properties of spore coat proteins (Aronson & Fitz-James, 1976), little is known of their function. Gould *et al.* (1970) suggested that the bacterial spore coat consisted of the two major proteins, disulphide-rich protein and alkali-soluble protein, both of which were located in the outer coat region of spores, and that alkali-soluble protein was involved in protecting the spores from enzymes but not in determining heat or radiation resistance. Labbe *et al.* (1978) reported that extraction of the coat protein with alkaline DTT from *Cl. perfringens* spores rendered the spores sensitive to lysozyme. These findings imply that lysozyme can penetrate the altered spore coat to reach the underlying cortex. In other words, alkali-soluble coat protein plays a role in the reinforcement of the coat as a permeability barrier. In a previous paper (Ando, 1975), we reported that H-spores of *Cl. perfringens* were sensitive to germination by lysozyme, but not Ca-spores. Although we have not yet studied the chemical properties of the alkali-soluble protein of the organism in detail, the protein was caused to precipitate with Ca^{2+} , Mg^{2+} and other divalent cations probably due to the formation of an associated complex (Y. Ando & T. Tsuzuki, unpublished data). If we assume that H- and Ca-spores comprise spore coat proteins as a dissociative and an associative form, respectively, the permeability of these spores to lysozyme would be explicable by the dissociation and association of the DTT-alkali-soluble protein. However, most of the carboxyl groups in the coat protein of H-spores seemed to have existed in an un-ionized form, because H-spores germinated so poorly in the KCl medium, as did native spores at pH 4. In fact, H-spores were transformed by incubation with a sodium phosphate buffer from the protonated form to a dissociated one, i.e., Na-spores, which are sensitive to lysozyme (Y. Ando & T. Tsuzuki,

unpublished data). Moreover, spores loaded with Ca^{2+} and other divalent cations still retained their colloidal charges and ability to germinate as well (Table 1). It is, therefore, reasonable to suppose that only a part of the DTT-alkali-soluble protein that is associated with Ca^{2+} may be responsible for preventing lysozyme from penetrating the spore coat. Nevertheless, the chemical modification of carboxyl groups in the spore coat caused alteration of the alkali-soluble protein to an alkali-insoluble one, thus yielding an altered spore coat having much reduced negative charge and impermeable to lysozyme as well as inorganic germinants (Y. Ando & T. Tsuzuki, unpublished data).

In view of the fact that bacterial spores behave as cation-exchangers (Alderton & Snell, 1963), the cation exchange capacity of the spores must be closely associated with the fixed, negatively charged carboxyl groups in the spore coat protein. The distribution of exchangeable cations such as Ca^{2+} or Sr^{2+} in the coat or subcoat region has been reported elsewhere (Tsuzuki & Ando, 1982). The reduced negative colloidal charges of spores loaded with various divalent cations might be the result of binding of the spore coat with divalent cations that can neutralize the negative charge (Table 1). The spore coat has properties very similar to those of a cation-exchange resin. It adsorbs more cations than anions from its environment due to the presence of fixed anions according to the Donnan equilibrium. Cation-exchange resin also shows swelling effects which are influenced by ionic strength. In the case of the ionic germination of the spores, coat swelling is considered to occur as soon as spores are suspended in the KCl medium, and thereafter the adsorbed K^+ will initiate germination in such a way as has been reported previously (Ando, 1979).

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