Relationship Between Hemagglutinin and Sialidase from
Clostridium perfringens CN3870: Gel Filtration of Mutant
and Revertant Activities

JULIAN I. ROOD* AND RUSSELL G. WILKINSON
School of Microbiology, University of Melbourne, Parkville, Victoria, Australia 3052

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Gel filtration of supernatant fluids, from the wild-type Clostridium perfrin-
gens, strain CN3870, and several of the mutants and revertants derived from
this strain, showed that these mutants failed to produce detectable amounts of
the I and II forms of both hemagglutinin and sialidase activities. However, they
still produced sialidase III activity. The revertants tested had regained the
ability to produce approximately wild-type levels of the I and II forms of both
activities. These results show that there is a direct relationship between the
production of the I form of hemagglutinin and sialidase activities and the
production of the II form of these biologically active proteins. Models that
explain the genetic basis for these results are discussed.

In the preceding publication (4) it is shown that the wild-type Clostridium perfrin-
gens, strain CN3870, produced three mucopolysaccharide N-acetyleneuraminylhydrolase (EC 3.2.1.18) (sialidase or neuraminidase) enzymes
designated as sialidases I, II, and III in order of decreasing molecular weight. Sialidase I and II activities were associated with hemagglutinin I
and II activities, respectively. Gel filtration, ion exchange chromatography, and polyacryl-
Amide gel electrophoresis failed to separate sialidase I activity from hemagglutinin I activity or sialidase II activity from hemagglutinin II activity. It was suggested (4) that, for both the I and II forms, hemagglutinin and sialidase activities were associated together in protein complexes. It was not known if there was any
relationship between the I and II complexes. In an earlier publication (3) we reported the isolation of a number of mutants, derived from
strain CN3870, that produced reduced levels of hemagglutinin and sialidase activities. From
one of these mutants, CM165, revertants that regained the ability to produce active hemagglutinin and sialidase were isolated.

It was decided to examine the gel filtration behavior of the activities present in several of
these mutants and revertants with the aim of identifying the specific hemagglutinin and sial-
idase activities affected by the mutations and investigating the relationship between the I
and II hemagglutinin-sialidase complexes. This paper reports the results of these investigations.

MATERIALS AND METHODS

Bacteria and materials. All mutant and revertant strains used in these studies were derived from
C. perfringens strain CN3870. The growth, maintenance, derivation, and phenotype of all strains, as
well as preparation of media, were as previously described (3). MacLennan broth (MB) was used in-
stead of fructose-Trypticase soy broth for reasons previously discussed (3). All reagents and materials
were from sources previously described (3, 4).

Estimation of hemagglutinin and sialidase activ-
ities and protein. Hemagglutinin and sialidase activities were estimated as previously described (2)
except that, unless otherwise stated, the sialidase incubation was carried out at pH 5.5. The number of hemagglutinin units was equal to the reciprocal of
the end point dilution and was expressed as the
number of units per milliliter of sample. One unit of
sialidase activity was defined as the amount of en-
zeyme required to hydrolyze 1 μmol of N-acetyleneu-
raminidamic acid per min from α1-acid glycoprotein un-
der the conditions of the assay. The protein content of column fractions was followed by determining the
absorbance at 280 nm. All spectrophotometric deter-
minations were made using a Zeiss PMQ II spectrophotometer.

Gel filtration on Sephadex G-200. Samples (5 ml)
of supernatant fluid were applied to and eluted from
Sephadex G-200 as previously described (4). The
column was eluted at 4°C, at a flow rate of 18 ml/h,
with tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.02 M, pH 7.2) containing 0.15 M KCl.

Preparation of supernatant fluid. For each
strain, 2 ml of a 2-h MB culture was subcultured into

1 Present address: Food Research Institute, University of
Wisconsin, Madison, Wis. 53706.
15 ml of MB medium and incubated for 6 h at 37 C.
After centrifugation for 15 min at 1,500 × g, the supernatant fluid was removed and treated as required.

**RESULTS**

It was previously shown (4) that sialidases I and II were inactive at pH 4.0 in 0.2 M potassium acetate buffer. Under these conditions sialidase III activity was stable and could therefore be assayed in the presence of the other two enzymes. Supernatant fluids from MB cultures of the strains examined in the following experiments were assayed for sialidase activity in 0.2 M potassium acetate buffer at pH 5.5 and 4.0. The results (Table 1) show that the wild-type CN3870 and revertants CM207, CM211, and CM213 had 4 to 10 times more sialidase activity at pH 5.5 than at pH 4.0, indicating that the major sialidase activities present were probably sialidases I and II, not sialidase III. The hemagglutinin reduced mutants CM156, CM165, and CM209 (an Spo+ revertant derived from strain CM165) all had sialidases that were only one to two times more active at pH 5.5, indicating that the major sialidase activity in these strains was probably sialidase III. Strains CN3870, CM211, and CM213 had higher levels of sialidase activity, at pH 4.0, than the other strains tested.

The gel filtration behavior of activities from each of these strains was examined by applying 5 ml of supernatant fluid, from MB cultures, directly onto Sephadex G-200. Prolonged incubation for up to 8 h was necessary to detect the low levels of sialidase activity present in column fractions derived from several strains. This necessitated the addition of 0.08% (wt/vol) sodium azide to the incubation mixtures to prevent microbial growth. Sialidase activity from all strains was assayed at pH 5.5, and fractions derived from the following strains were also assayed at pH 4.0 to detect sialidase III activity. The latter strains were: the wild-type CN3870; a group C mutant (2), CM165; a group A revertant, CM213; and a group B revertant, CM207. Fractions derived from strains CN3870, CM211, and CM213 were also assayed for hemagglutinin activity. This was not possible with CM207, the only other strain tested here that produced detectable hemagglutinin activity, as its activity was too low to be detected because of dilution during chromatography.

The supernatant fluid from an MB culture of strain CN3870 had approximately the same levels of hemagglutinin and sialidase activities as the fructose-Trypticase soy broth supernatant fluid examined previously by chromatography on Sephadex G-200 (4). However, the distribution of both activities between the I and II forms was different. The elution profile of the latter showed that the predominant activities were hemagglutinin I and sialidase I (4). In contrast, the elution profile of the MB supernatant fluid showed that the major sialidase activity was now sialidase II, with a shoulder of sialidase I, and that hemagglutinin was now spread in a plateau of activity covering the hemagglutinin I and II regions (Fig. 1). The explanation for these results is not known and these effects were not studied further. When the fractions obtained in the MB experiment were assayed for sialidase activity at pH 4.0, a small peak of sialidase III activity was detected, thus demonstrating the presence of this enzyme in supernatant fluids derived from strain CN3870.

**Chromatography of supernatant fluid from an MB culture of strain CM165, a mutant producing low levels of sialidase activity but no detectable hemagglutinin activity, showed that this strain did not produce detectable levels of the I and II forms of either activity (Fig. 2). However, sialidase III activity was still present, as confirmed by the coincidence of the activity profiles obtained at pH 5.5 and 4.0. Strains CM156 and CM209, both with hemagglutinin and sialidase phenotypes identical to strain CM165, gave elution profiles similar to that obtained from the latter strain, except that these fractions were not assayed for sialidase activity at pH 4.0. In all three strains sialidase III activity was the only sialidase activity detected.

**Chromatography of supernatant fluid from MB cultures of strain CM213, a revertant that regained the ability to produce hemagglutinin and sialidase activities, showed that this strain produced approximately wild-type levels of the I and II forms of both activities. Sialidase III**

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**Table 1. Sialidase activities (at pH 5.5 and 4.0) from various strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent strain</th>
<th>Relevant phenotype</th>
<th>Sialidase activity (a)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>pH 5.5</td>
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<tr>
<td>CN3870</td>
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<tr>
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<td>CM165</td>
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<td>0.46</td>
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(a) Supernatant fluids, prepared from MB cultures as in Materials and Methods, were assayed for sialidase activity in 0.2 M potassium acetate buffer at pH 5.5 and 4.0. Activities are expressed as units per milliliter of supernatant fluid at the pH of the assay.

(b) See Rood and Wilkinson (9).
activity was also present (Fig. 3). Both hemagglutinin and sialidase activities were resolved into a major peak of the II form, together with a smaller peak of the I form. Strain CM211, which had a hemagglutinin and sialidase phenotype identical to strain CM213, gave similar elution profiles except that these fractions were not assayed for sialidase activity at pH 4.0. Strain CM207, a revertant producing hemagglutinin and sialidase activities at levels significantly lower than wild type, also gave an elution profile similar to that of strain CM213, except that there was a shoulder of sialidase activity at pH 5.5 which corresponded to the peak of sialidase III activity observed at pH 4.0 (Fig. 4).

**DISCUSSION**

The results presented in this paper show that the hemagglutinin-reduced mutants CM165 and CM156 failed to produce detectable amounts of the I or II active forms of either hemagglutinin or sialidase. However, they still produced sialidase III activity, although the levels were less than those of the wild-type CN3870. Strains CM211 and CM213, both derived from strain CM165, were revertants that regained the ability to produce hemagglutinin and sialidase activities. They also produced approximately wild-type levels of sialidase III activity. Gel filtration experiments revealed that these strains had regained the ability to produce the I and II forms of both activities in approximately the same proportions as in the wild type. Another revertant, CM207, produced more hemagglutinin and sialidase activity than its hemagglutinin-reduced parent but not as much activity as the wild type. Gel filtration showed that it also regained the ability to produce both sialidase I and II activities. It is likely that the other partially reduced mutants and revertants described previously (3) also produced reduced levels of both active forms.

The phenotypes of the strains reported previously (3) were designated on the basis of their
FIG. 2. Gel filtration on Sephadex G-200 of sialidase activity from supernatant fluid from an MB culture of strain CM165. Five milliliters of supernatant fluid (containing 0.079 and 0.043 units of sialidase activity at pH 5.5 and 4.0, respectively) was applied to and eluted from Sephadex G-200. Fractions were collected and assayed for sialidase activity in 0.2 M potassium acetate buffer at pH 5.5 (○) and at pH 4.0 (○), and the absorbance was read at 280 nm (×).

overall hemagglutinin and sialidase activities. The identification of different molecular forms of these activities (4), together with the experiments reported in this paper, now enables a more precise phenotypic designation of the strains studied in this report. All the residual sialidase activity from strains CM156, CM165, and CM209, which were originally designated Hgn-Sldred, was in the sialidase III form. These strains are therefore now designated as HgnI-SldI HgnII-SldII-SldIIIred (Table 2).

The results, reported both in this paper and in an earlier publication (3), show that a single point mutation can affect the expression of both hemagglutinin and sialidase I and hemagglutinin and sialidase II activities concomitantly. This mutation also affects the expression of sialidase III activity but not to the same extent. Strain CM171 was the only strain previously designated (3) as Hgn-Sld-. It is possible that this strain carries two mutations, one in a gene affecting the expression of hemagglutinin and sialidase I and II activities and the other in a separate gene affecting the expression of sialidase III activity only. The biochemical data reported previously (4) and the results presented here all provide evidence that sialidase III is not a monomeric form of the I and II proteins. It would be of interest to attempt the isolation of sialidase III mutants, by screening for mutants producing supernatant fluids that had no sialidase activity at pH 4.0 in acetate buffer, to see if such mutants produced altered forms of the I and II activities.

Two models are proposed that could explain the genetic basis for the observations reported in our studies. In these models it is assumed that sialidase III is not structurally related to the I and II proteins. For simplification, it will be initially assumed that only one of the I and
II molecular forms is produced. Hypotheses will then be presented to account for the presence of two forms of both hemagglutinin and sialidase activities.

The first model proposes that there is a gene, mutations in which coordinately affect the expression of the separate structural genes coding for structurally distinct, noninteracting, hemagglutinin and sialidase proteins. The hemagglutinin reduced mutants would have arisen through mutations in this gene, rather than in the structural genes, because mutations in the latter would result in mutants that are only altered in the expression of one activity. The fact that Hgn 'Sld' mutants were not found in these studies and that Hgn 'Sld' strains were not reported previously (1) may provide evidence against this model, although the former case may reflect differences in the susceptibility of the various genes to mutagenesis by N-methyl-N-nitro-N'-nitrosoguanidine. The model predicts that the hemagglutinin reduced mutants would not produce inactive hemagglutinin or sialidase proteins.

The second model proposes that hemagglutinin and sialidase activities are associated in a protein complex. The term complex does not distinguish between an aggregate of different polypeptides, which includes structurally distinct hemagglutinin and sialidase subunits coded for by separate structural genes, or a single polypeptide that contains both activities and is coded for by a single structural gene. The model suggests that the hemagglutinin-reduced mutants would have arisen through mutations in a structural gene coding for an essential subunit of the complex such that either no protein at all was produced or the protein was altered so that it was inactive. It predicts that hemagglutinin and sialidase activities cannot be separated by biochemical separation techniques. If the complex is made up of hemagglutinin and sialidase subunits, then it is necessary to postulate further that it is only active...

![Fig. 3. Gel filtration on Sephadex G-200 of hemagglutinin and sialidase activities from supernatant fluid from an MB culture of strain CM213. Five milliliters of supernatant fluid (containing 2.3 and 0.23 units of sialidase activity at pH 5.5 and 4.0, respectively, and 1,800 units of hemagglutinin activity) was applied to and eluted from Sephadex G-200. Fractions were collected and assayed for sialidase activity in 0.2 M potassium acetate buffer at pH 5.5 (○) and pH 4.0 (○) and hemagglutinin activity (●), and the absorbance was read at 280 nm (×).](http://jb.asm.org/.../10.1128/JB.126.11.847-855.1976)
FIG. 4. Gel filtration on Sephadex G-200 of sialidase activity from supernatant fluid from an MB culture of strain CM207. Five milliliters of supernatant fluid (containing 0.56 and 0.078 units of sialidase activity at pH 5.5 and 4.0, respectively) was applied to and eluted from Sephadex G-200. Fractions were collected and assayed for sialidase activity in 0.2 M potassium acetate buffer at pH 5.5 (○) and pH 4.0 (□), and the absorbance was read at 280 nm (×).

TABLE 2. Amended phenotypic properties of various strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>CN3870</td>
<td>HgnI&lt;sup&gt;+&lt;/sup&gt; SldI&lt;sup&gt;+&lt;/sup&gt; HgnII&lt;sup&gt;+&lt;/sup&gt; SldII&lt;sup&gt;+&lt;/sup&gt; SldIII&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<tr>
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<sup>a</sup> All other phenotypic properties were as previously described (3).
<sup>b</sup> —, Not tested.

when they are combined and that the subunits themselves are inactive. Otherwise, it would not be possible for a mutation in a structural gene coding for a hemagglutinin subunit to affect sialidase activity, and vice-versa. It is also necessary to postulate that, if a mutation results in the production of a subunit that is altered such that it is inactive even when complexed with the normal subunit, then this also results in the formation of a complex that is not active for either activity. The mutations reported could have been in either of the structural genes. They may have resulted in the production of either an inactive complex or only one of the normal inactive subunits.

A simpler version of the second model is that the complex is in fact a single polypeptide, having both hemagglutinin and sialidase activities, that may or may not act by a common active site. The biochemical data reported in the preceding paper (4) support either version of the second model, rather than the first model, since the separation techniques used failed to separate the respective hemagglutinin and sialidase activities.

The presence of two forms of hemagglutinin

The biochemical data reported in the preceding paper (4) support either version of the second model, rather than the first model, since the separation techniques used failed to separate the respective hemagglutinin and sialidase activities.

The presence of two forms of hemagglutinin
and sialidase can be incorporated by postulating either that the hemagglutinin and sialidase I complex is a polymer of the hemagglutinin and sialidase II complex, or that they are not structurally related. If the former is the case, then a mutation resulting in an inactive II complex must also affect the I complex, assuming that the same active sites are involved in each complex. Alternatively, if the complexes are not structurally related, then their expression must be concomitantly influenced by mutations in another gene. The occurrence of mutants that produce only one active complex is therefore postulated. No such mutants were observed, although it is not certain that they would have been detected by the screening techniques used.

In summary, the results at this stage are most compatible with the theory that hemagglutinin and sialidase activities reside on the one protein, HgnSldII, that aggregates to form the HgnSldI protein, which retains both activities. The alternative model is not completely ruled out by our results. Neither of the models presented here explains the relationship between the production of hemagglutinin and sialidase I and II activities and either sporulation, the production of phospholipase C activity, or the production of sialidase III activity. They also do not attempt to explain how the distribution of both hemagglutinin and sialidase activities between the I and II forms is controlled. Some, or all, of the above effects may be due to either extragenic effects of an altered gene product or polarity effects in genes adjacent to the gene carrying the mutation. The development of a more sophisticated genetic system for studying this organism should provide a means of investigating these relationships.

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LITERATURE CITED