Glycoconjugate Journal 15, 769–775 (1998)

Effect of cysteine modifications on the activity of the 'small' *Clostridium perfringens* sialidase

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The 'small' (43 kDa) sialidase of *Clostridium perfringens* is inhibited by low concentrations of mercury ions. For the investigation of possible functional roles of the enzyme's four cysteine residues at the amino acid positions 2, 282, 333 and 349, they were separately altered to serine by site-directed mutagenesis. The four mutant sialidases expressed in *E. coli* and purified by metal chelate chromatography were markedly reduced in specific activity when compared to the wild-type enzyme but with the exception of C282S exhibited similar K_M-values indicating an unchanged mode of substrate binding. The substrate specificity was also conserved for C2S, C282S, and C333S. Only the C349S sialidase exhibited a higher relative activity with colominic acid and the α2,6-linked sialic acid of sialyllactose compared to the α2,3-linked isomer than the other mutants. Chemical modifications with the thiol-blocking reagents *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (pCMB) and $HgCl_2$ had little effect on the C282S sialidase, e.g., 6% inhibition by 5 mM NEM compared to reductions in activity between 65 and 90% for the wild-type and other mutant enzymes, supporting the idea that among the enzyme's cysteines, Cys-282 has the highest structural or functional significance. The results also explain the higher mercury tolerance of *Salmonella typhimurium* and *Clostridium tertium* sialidases, which have the positions equivalent to Cys-282 altered to Val and Thr, respectively, indicating that the thiol group of Cys-282, despite being situated near the active site, is not involved in catalysis.

***Keywords*: sialidase, mutagenesis, cysteine modification, mercury tolerance, modulation of kinetic properties**

Introduction

Sialidases (*N*-acylneuraminosyl glycohy dro lases, EC 3.2.1.18) hydrolyse α-glycosidically bound sialic acids [1], which are frequently found as terminal constituents of oligosaccharides, glycoproteins and glycolipids in higher animals and some microorganisms [2]. These enzymes, however, are distributed not only throughout the metazoan animals of the deuterostomate lineage, but also occur in some protozoa, viruses, fungi and bacteria [3]. Whereas the different kinds of sialidases in animals play an important role in the turnover of sialoglycoconjugates, thereby influencing the lifetime of cells and molecules [4, 5], the biological function of the microbial enzymes has not been finally clarified, especially since sialidases are sometimes produced only by single strains of a bacterial species [6]. Remarkably, the sialidases are often found in microorganisms which live in close contact with an animal host, whereby the enzyme may serve as a colonization and virulence factor, or as a tool for nutrition [7, 8]. The nucleotide sequences of several sialidases have been investigated and similarities were detected supporting the hypothesis of a common sialidase origin [9].

The 'small' (43 kDa) sialidase of *Clostridium perfringens* A99 was the first bacterial sialidase for which several amino acids have been exchanged by site-directed mutagenesis in order to investigate their structural or functional importance [10]. Strikingly, this enzyme was inhibited by low concentrations of $HgCl_2$ [11], giving a hint of a possible participation of one or more of its cysteines in substrate binding, catalysis or structure stabilization. This sialidase possesses four cysteines at positions 2, 282, 333 and 349. In order to deduce the significance for structure and activity of the individual cysteine residues, they were altered separately to serine. The purified wild-type (WT) and mutant enzymes were characterized and their changes in activity after chemical modification of the thiol groups were analyzed.

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Materials and methods

Bacterial strains, vectors and media

Escherichia coli JM 101 [12] was purchased from Pharmacia (Freiburg, Germany), while *E. coli* BL21(DE3)pLysS [13] used for the expression of the sialidases was a gift of Dr. Constantin Vorgias (EMBL, Hamburg, Germany). The bacteriophage vector for cloning and sequencing (M13mp19) [14] was from Boehringer (Mannheim, Germany), and the expression vector pQE-10 (QIAexpressionist Kit type IV) was from QIAGEN (Hilden, Germany).

E. coli was propagated at 37 °C in Luria Bertani broth [15] or on 1.8% agar media. Alternatively, 2 × YT broth or M9 minimal broth were used, and after electroporation, cells were grown in SOC medium [15]. Media were supplemented with 34 mg l^{-1} chloramphenicol (Sigma, Deisenhofen, Germany) for the selection of the pLysS-vector and with 100 mg l^{-1} ampicillin (Boehringer, Mannheim, Germany) and 0.1 mM isopropylthio-β-D-galactoside (IPTG; Serva, Heidelberg, Germany) for the selection of the pQE-10 vector.

Site-directed mutagenesis and expression of the mutant sialidases

The wild-type sialidase structural gene was obtained by amplifying the original gene [16] from chromosomal DNA of *C. perfringens* strain A99 by PCR with primers introducing *Bam*HI-cleavage sites at both ends of the product in order to facilitate cloning [17]. Then the PCR product of 1.16 kb was inserted into the phage vector M13mp19 and single-stranded DNA was prepared according to [15]. The appropriate strand was selected by hybridization with the ^{32}P-labeled non-template PCR primer. Site-directed mutagenesis was performed with the 'Sculptor™ *in vitro* mutagenesis system' (Amersham, Braunschweig, Germany) by using the 5′-phosphorylated primers (Dr. W. Kullmann, Institut für Zellbiologie und Klinische Endokrinologie, Hamburg, Germany) GAG GAT CCA TCC AAC AAA AAC AAT AC for the C2S-mutation, GGC TCT GGA TCG CAG GGT TCC TTT ATT for C282S, GTT CAA GAA ATA TCG ATT CCT TAT CC for C333S and GT GGC TAT TCT TCT CTA TCA TTT AAA for C349S (exchanged nucleotides underlined). These primers were also used to select the mutants by colony hybridization [15]. Each mutation was confirmed by sequencing of the final DNA-product with Sequenase-Kit 2.0 (USB, Cleveland, USA).

The double-stranded DNA of the phages was prepared from the host cells [15] and digested with *Bam*HI. The mutated sialidase genes were inserted into the expression vector pQE-10. *E. coli* BL21 (DE3) pLysS was transformed with these constructs by electroporation. After incubation on selective media, the sialidase-producing clones were selected by a qualitative activity assay with 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac) (Sigma, Deisenhofen, Germany) or by proving the presence of an 1.16 kb insert after plasmid-preparation [15].

Enzyme purification

E. coli clones expressing the mutant sialidases were grown overnight in 2 ml or 20 ml cultures at 37 °C. After 14–18 hrs of growth, the cells were harvested by centrifugation (6,000 × **g**, 10 min, 4 °C) and resuspended in 0.2 ml or 2 ml of lysozyme buffer (25 mM Tris, 2 mM EDTA, pH 8.0, with 5 mg lysozyme ml^{-1}), respectively. The suspension was incubated for 30–60 min at 37 °C for release of the sialidase. After centrifugation (13,000 × **g**, 10 min, 4 °C), the enzyme was found in the supernatant, where it was detected by activity measurement and SDS-polyacrylamide gel electrophoresis according to Laemmli [18].

The expression system pQE-10 added a six histidine-containing affinity tag (His_6) to the N-terminus of the protein, thus allowing the purification of the sialidase by affinity chromatography on Ni-nitrilo-tri-acetic acid agarose (Ni-NTA Spin, Kit; QIAGEN, Hilden, Germany). The spin columns were equilibrated with 600 µl of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, for 2 min at 2,000 rpm. Up to 600 µl of the lysozyme supernatant were applied to the column, which was washed once with 600 µl of the equilibration buffer and twice with 50 mM sodium phosphate buffer, pH 6.0, containing 300 mM NaCl. The sialidase was eluted with 2 × 200 µl 50 mM sodium phosphate buffer, pH 6.0, containing 300 mM NaCl and 250 mM imidazole. After measuring the sialidase activity, protein content, and purity in SDS-PAGE, the fractions containing pure enzyme were pooled and stored at 4 °C. For a longer storage 0.01 M sodium azide was added.

Sialidase and protein assays

In the qualitative sialidase assay, *E. coli* colonies were sprayed with MU-Neu5Ac. Those expressing sialidase activity were identified by their blue-white fluorescence under UV-light (360 nm) [16]. Sialidase activity was quantified during enzyme purification and with the thiol-modified sialidases by incubating 10 µl enzyme solution with 80 µl 0.1 M sodium acetate buffer, pH 6.1, and 10 µl of 1 mM MU-Neu5Ac for 10 min at 37 °C (standard assay conditions). If necessary, the enzyme sample was diluted with a 50 mM maleic acid buffer, pH 6.1, containing 1 mg ml^{-1} bovine serum albumin and 0.02% sodium azide. Free 4-methylumbelliferone (MU) was measured in a M1000 fluorimeter (Perkin-Elmer, Überlingen, Germany) using excitation at 365 nm and emission at 450 nm. The instrument was calibrated with MU solutions [19]. One unit was defined as 1 µmol of product formed per min.

In order to determine the influence of pH on catalysis, the activity was measured at 0.1 mM MU-Neu5Ac concentration in a buffer system with a constant ionic strength of 0.1 mol l^{-1} containing 0.05 M acetic acid, 0.1 M triethanolamine and 0.05 M 2-(*N*-morpholino)ethanesulfonic acid [20]. The pH was adjusted with hydrochloric acid

or tetraethylammonium hydroxide in the pH-range of 3.0 to 9.0 in steps of 0.5.

Kinetic experiments were performed at substrate concentrations of 1.0 mM, 0.5 mM, 0.2 mM, 0.1 mM and 0.05 mM MU-Neu5Ac by incubation for 10 min at 30°C with the buffer system described above [20] adjusted to pH 6.5-K_M and V_{max} were calculated by fitting the data to the Michaelis-Menten equation by non-linear regression with the program Enzfitter (Biosoft, Cambridge, UK).

The substrate specificity was tested with α2,3- and α2,6-sialyllactose [21], colominic acid (α2,8-polysialic acid; Sigma) and native bovine submandibular gland mucin (BSM) [22] by determining the amount of liberated sialic acids after incubation with 5 µl enzyme solution (0.1–0.4 mU), 50 µl 0.2 M sodium acetate buffer, pH 6.1, and 45 µl sialoglycoconjugate solution (final concentration of sialic acids 1 mM) for 20 min at 30 °C, according to Reuter and Schauer [22].

Protein concentrations were determined by the Bio-Rad micro-protein assay (Bio-Rad, München, Germany) using different concentrations of bovine serum albumin (BSA; Sigma, Deisenhofen, Germany) as standard.

Chemical modification of the sialidases

The thiol modifying agents NEM, pCMB and $HgCl_2$ were used to measure the rate of inactivation of the mutant sialidases in comparison to the wild-type enzyme (WT) [17] and the sialidase of *Salmonella typhimurium* (Sigma, Deisenhofen, Germany).

For determining the reagent concentration needed for 50% enzyme inactivation, the following concentrations were tested: 0, 0.5, 1, 2, 5, and 10 mM NEM; 0, 0.01, 0.1, 1, 10, 100, and 1000 µM pCMB and 0, 0.01, 0.05, 0.1, 1, 10, and 100 µM$HgCl_2$. Reactions were performed in siliconized tubes for 15 min at 25 °C in 50 mM maleic acid buffer, pH 6.0 (NEM), or in 0.1 M sodium acetate buffer, pH 6.1 (pH 5.1 in the case of the *S. typhimurium* sialidase) for 10 min at 30 °C (pCMB, $HgCl_2$) in the presence of 0.1 mM MU-Neu5Ac. At this low substrate concentration significant protection against modification would not be expected.

For investigation of the time-dependence of inactivation, the different sialidases at protein concentrations of 0.007 µM (C2S), 0.7 µM (C282S), 0.005 µM (C333S), and 0.0014 µM (C349S) in 50 mM maleic acid buffer, pH 6.1, were incubated with 5 mM NEM in siliconized tubes. Samples of 10 µl were withdrawn at regular intervals and the sialidase activity was measured with the standard assay. The relative activity data were fitted to theoretical models describing the decrease of activity that is caused by the modification of one or two cysteine residues [23] with the program Enzfitter. In the case of residual activity after modification, two models were applicable:

(i) Model 1: The modification of one cysteine residue is responsible for the inactivation.

$$A/A_0 = (1 - F_1)e^{-k_1 t} + F_1,$$

where A/A_0 represents the relative activity at time t, F_1 is the relative residual activity of the modified enzyme, and k_1 is the rate of constant for the loss of activity.

(ii) Model 2: The modification of two cysteine residues is responsible for inactivation.

$$A/A_0 = F_3 + (1 - F_1 - F_2 + F_3)e^{-(k_1 + k_2)t} + (F_1 - F_3)e^{-k_2 t} + (F_2 - F_3)e^{-k_1 t}$$

Modification of one group leads to the activity F_1, that of the other to F_2, that of both residues to the residual activity F_3. The velocity constants of the two inactivating reactions are k_1 and k_2.

Results and discussion

Characterization of the native wild-type and mutant sialidases

Each of the four cysteine residues was successfully replaced by serine, which was confirmed by DNA-sequencing. In contrast to former experiments with the wild-type sialidase [11], the recombinant sialidases were purified by affinity chromatography to apparent homogeneity in SDS-polyacrylamide gel electrophoresis (data not shown) [17]. The use of Ni-NTA spin columns reduced the time of this one-step purification to about 30 min, which resulted in highly active enzyme preparations with little protein denaturation. Final yields of about 1 mg protein from 1 litre of culture medium were obtained with a specific activity of 270 U mg^{-1} for the WT-enzyme. The presence of the extra six-histidine residues at the N-terminus did not appear to effect the catalytic properties, as has been shown for the wild-type sialidase [17].

From the kinetic data (Table 1), the K_M-value for C282S was approximately double that of the wild-type and other mutant, implying a reduced substrate affinity for this mutant sialidase. In contrast, no significant difference in the K_M-values compared to those of the WT-enzyme was observed for the C2S, C333S, and C349S sialidases. Therefore, only Cys-282 might be regarded as being part of or near to

Table 1. *Kinetic data of the four mutant sialidases compared to the wild-type enzyme*

Enzyme	*K_M (mM)*	*mol. activity (1 per min)*	*rel. V_{max} (%)*
WT	0.17	144000	100
C2S	0.17	1400	1
C282S	0.41	60	0.04
C333S	0.19	600	0.40
C349S	0.16	53400	37

Activity was measured with the substrate MU-Neu5Ac at pH 6.0. Results shown are means of three experiments.

Table 2. *Substrate specificity of the mutant sialidases in comparison to the wild-type enzyme*

Enzyme	*Relative activities [%] with the substrates*			
	α2,3-sialyllactose	α2,6-sialyllactose	Colominic acid	BSM
WT	100	18	3.5	5.0
C2S	100	17.5	4.0	4.0
C282S	100	16	4.5	3.0
C333S	100	16	4.0	4.5
C349S	100	35	13.0	6.0

The hydrolytic rates at 1 mM sialic acid concentration are shown relative to the data obtained with α2,3-sialyllactose which were set to 100%. Results shown are means of three experiments.

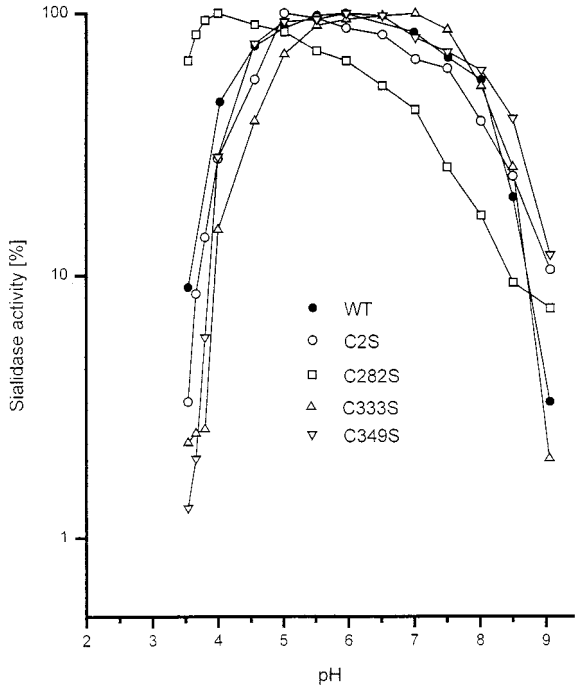

Figure 1. Sialidase activity of the wild-type and four mutant enzymes at different pH values. The relative sialidase activities are shown relative to the pH optimum value for each enzyme form which is set as 100%. The results are means of three experiments.

the active site. All mutant enzymes were markedly reduced in molecular activities, especially C282S, that exhibited only 0.04% of the rate of the WT-form (Table 1). However, sialidase activity was still detectable for all enzymes, indicating that these mutations did not alter the 3D-structure in a way that prevented catalysis.

The substrate specificity of the C2S, C282S, and C333S sialidases was comparable to that of the wild-type form when tested with α2,3- and α2,6-sialyllactose, α2,8-polysialic acid, and native bovine submandibular gland mucin (Table 2). Interestingly, the C349S sialidase exhibited a two-fold higher activity with α2,6-sialyllactose and a 3.7-fold higher activity with the α2,8-linked sialic acid of colominic acid relative to α2,3-sialyllactose as compared with WT-enzyme or the other mutants. Previous modeling experiments 24 suggested that C. perfringens sialidase probably exhibits a similar tertiary structure to that of *S. typhimurium* sialidase, in which Cys-344 is located below Tyr-342 [25]. This amino acid is assumed to interact with the substrate during catalysis by stabilizing an oxocarbonium intermediate of sialic acid [26]. Changes at Cys-349 of the *C. perfringens* sialidase may, therefore, cause conformational changes at the active site that influence the substrate specificity. A partially altered conformation caused by the C349S exchange has already been detected by the laser photo CIDNP (Chemically Induced Dynamic Nuclear Polarization) NMR approach and was predicted by the homology modeling of the wild-type and mutant sialidase structures [24]. Changes in substrate specificity were not observed with the other mutant sialidases or with the bovine submandibular gland mucin substrate, that contains mainly α2,6-linked sialic acids.

Testing the sialidase activities in the range of pH 3.0–9.0 revealed that the WT, C2S, C333S, and C349S enzymes exhibited broad pH optima between pH 5 and pH 7 with only minor deviations in pH dependence of the mutant sialidases when compared to the WT-form (Figure 1). Only the C282S mutant, however, showed a pH optimum shifted to pH 4. To rule out the possibility that this effect was influenced by denaturation at extreme pH values, the activities were measured and shown to be stable at pH 3.5 and 8.5 for at least 30 min (data not shown).

Chemical modification of the wild-type and mutant sialidases

Modifying the wild-type and mutant sialidases using different concentrations of NEM demonstrated that the C282S enzyme was inhibited far less than the other forms of the enzyme, losing only 6% of its activity during 15 min of incubation with 5 mM reagent. On the other hand, the WT, C2S, C333S, and C349S were inactivated by 65 to 90% under these conditions. Fifty percent inactivation was observed for the wild-type sialidase at 2.6 mM and
NEM for the C2S, C349S, and C333S enzymes at 2.9, 2.6 and 1.6 mM respectively. Cys-282 therefore, accounts for the major part of the inactivation by NEM-modification, the influence of the other cysteines being only minimal.

In order to further investigate the impact of the modification of these cysteines on activity, the kinetics of inactivation of the wild-type and the mutant enzymes C2S, C333S, and C349S were measured at 5 mM NEM (Figure 2). After modification the wild-type, C333S and C349S enzymes exhibited residual activities of 9, 2 and 4%, respectively, confirming that the SH-groups are not directly involved in catalysis. The best fits to the residual activity data of the

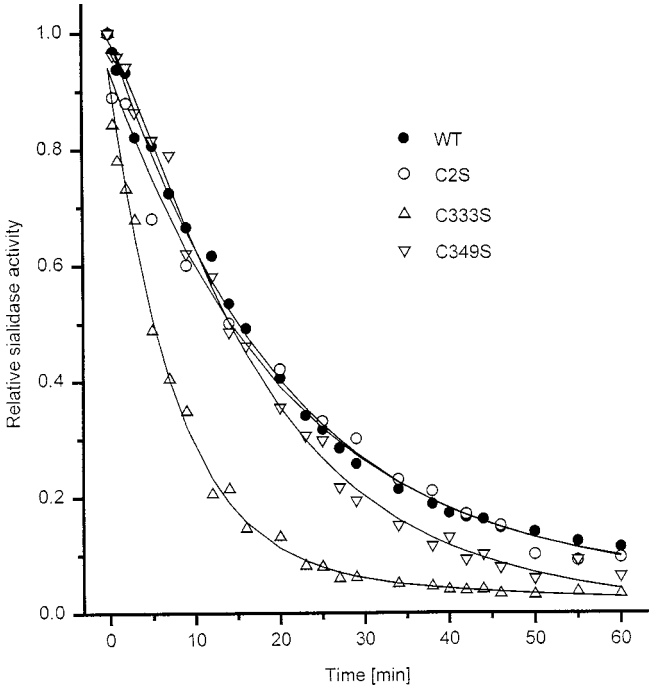

Figure 2. Inactivation of the wild-type and three mutant enzymes by 5 mM NEM. The relative sialidase activity at time 0 was defined as 1. The regression curves shown were calculated assuming model 2. The results are means of two experiments.

WT, C2S, C333S, and C349S enzymes were achieved with model 2, indicating that besides the modification of Cys-282, at least one additional cysteine participated in the inactivation, as was already suggested by the minor inactivation of the C282S sialidase. The similarity of the inactivation behavior of C2S with that of the wild-type form indicates that modification at cysteine 2 did not influence the activity. The C333S enzyme was inactivated even faster than the WT form. The removal of a modifiable group did not protect the sialidase but enhanced the rate of inactivation, possibly by influencing the conformation of the enzyme.

Modification of the sialidases by the charged reagent pCMB revealed that for achieving 50% inhibition a 1000-fold higher concentration was needed for C282S and a 5-fold higher concentration for C349S, whereas C2S and C333S behaved in the same way as the WT enzyme (Table 3). A similar pattern of inactivation was observed with the smaller reagent $HgCl_2$ (Table 3). This suggests that for both modifying agents Cys-282 and to a limited extent Cys-349 were responsible for inactivation. The modification of other bacterial sialidases revealed that the enzymes from *S. typhimurium* and *C. tertium* are even more resistant to inactivation by pCMB or $HgCl_2$ (Table 3), whereas the 'large' *C. perfringens* [11] and *C. chauvoei* [27] sialidases behave similarly to C282S when incubated with $HgCl_2$.

Table 3. *Concentrations of pCMB and of $HgCl_2$ needed for 50% inactivation of the natural and the mutated sialidases*

Enzyme	*pCMB*		*$HgCl_2$*	
	conc. (μM)	*relative conc.*	*conc. (μM)*	*relative conc.*
C. perfringens 'small'				
Wild-type	0.08	1	0.07	1
C2S	0.07	0.9	0.07	1
C282S	80	1000	9.5	140
C333S	0.09	1.1	0.05	0.7
C349S	0.4	5	0.11	1.6
S. typhimurium	540	6800	23	330
C. chauvoei	ND	ND	1.3	18
C. perfringens 'large'	ND	ND	5	75
C. tertium	ND	ND	5200	75000

The concentrations leading to 50% inhibition of the WT enzyme are set to 1 in order to better demonstrate the relative inhibitory capacity of the mercury agents on the different sialidases. Results shown are means of three experiments each. ND, not determined.

The experimental data presented herein indicates that in the 'small' *C. perfringens* sialidase the cysteine at position 282 has a greater structural and functional importance than the other cysteines. A cysteine residue is conserved at this position in most, but not in all bacterial sialidases (Figure 3), e.g., in the sialidase from *S. typhimurium* a valine is present and in the *C. tertium* enzyme a threonine is found. This is in agreement with the known tolerance of these sialidases towards pCMB and $HgCl_2$. This may indicate an adaptation of these bacteria to environmental requirements (e.g., soil). The higher resistance of the large *C. perfringens* sialidase cannot yet be explained. This enzyme has a cysteine at the position equivalent to Cys-282 but not at the position corresponding to Cys-333 in the native 'small' sialidase. This sequence pattern is also shared by the *Bacteroides fragilis* and *C. septicum* sialidases (Figure 3), which might therefore be assumed to exhibit comparable sensitivities towards $HgCl_2$. The naturally occurring substitutions of Cys-282 by valine and threonine in the *S. typhimurium* and *C. tertium* sialidases rule out the requirement of this thiol function for catalytic activity. However, within a distance of 0.4 nm from Val-282 of the *S. typhimurium* enzyme are situated Arg-246 and Arg-309, two residues that interact with the carboxyl group of the inhibitor 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid, and probably also sialic acid [25, 26].

Modifications at the equivalent site of the *C. perfringens* sialidase, Cys-282, either by mutagenesis or by thiol-modifying agents, can be expected to affect the interactions of the homologous Arg-245 and Arg-312 with the substrate, which may shift pH-optima and reduce the enzyme's activity.

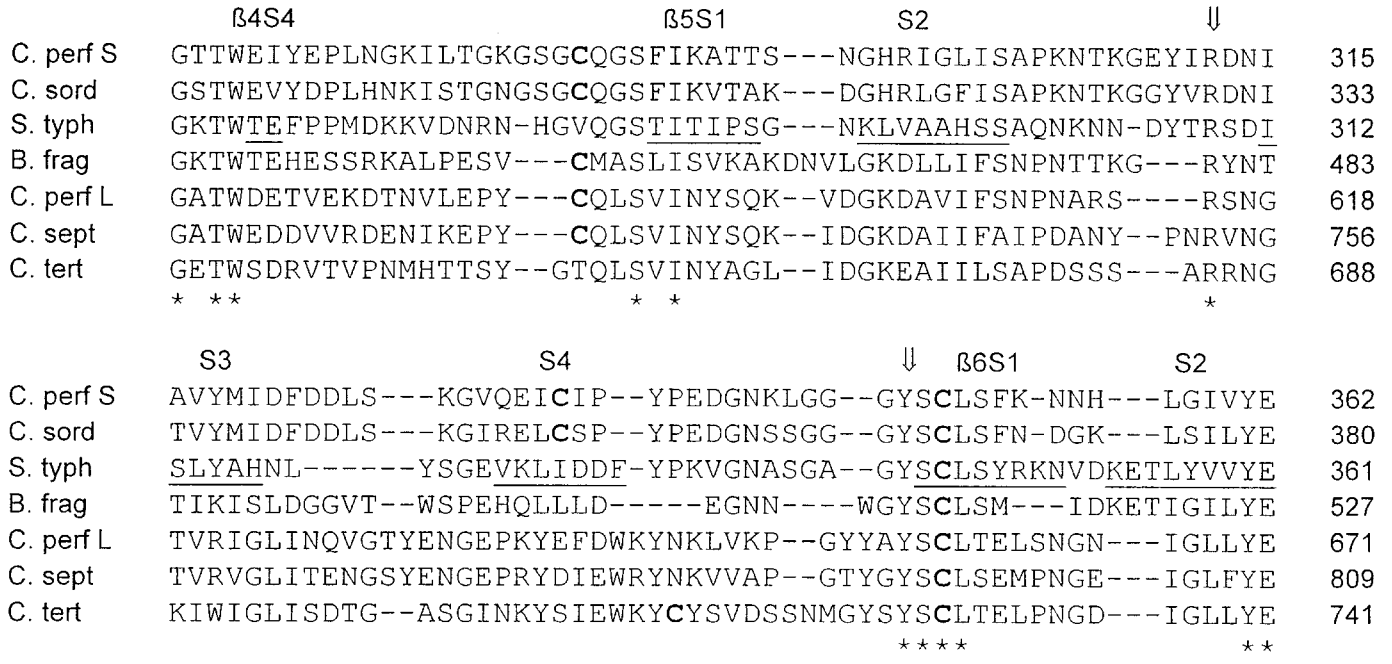

Figure 3. Alignment of the C-terminal parts of the amino acid sequences from various bacterial sialidases. The sialidase sequences from *Clostridium perfringens* ('small' [16], S, and 'large' [28], L, isoenzyme), *C. sordellii* [29], *Salmonella typhimurium* [25], *Bacteroides fragilis* [30], *C. septicum* [31], and *C. tertium* [EMBL Nucleotide Sequence Database accession No. Y08695] were aligned with the program Clustal W [32]. Cysteines are shown in bold print and *β*-strand regions of the *S. typhimurium* sialidase [25] are underlined and named above the alignment. Amino acids conserved in all sialidases shown are indicated by stars and the functional residues Arg-312 and Tyr-347 by arrows.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (grant Scha 202/19), the Sialic Acids Society (Kiel) with a doctoral fellowship (Susanne Kruse), and the Fonds der Chemischen Industrie.

References

1 Corfield AP, Michalski JC, Schauer R (1981) In *Sialidases and Sialidosis* (Tettamanti G, Durand P, DiDonato S, eds) Vol 4, pp 3–70. Milano: Edi Ermes.
2 Corfield AP, Schauer R (1982) In *Sialic Acids – Chemistry, Metabolism and Function* (Schauer R, ed) pp 5–50. Cell Biology Monographs Vol 10, Berlin: Springer Verlag.
3 Saito M, Yu RK (1995) In *Biology of Sialic Acids* (Rosenberg A, ed) pp 261–313. New York: Plenum Press.
4 Schauer R (1982) *Adv Carbohydr Chem Biochem* **40**: 131–234.
5 Schauer R (1983) In *Structural Carbohydrates of the Liver* (Popper H, Reutter W, Köttgen E, Gudat F, eds.) pp 83–97. Boston: MTP Press Ltd.
6 Popoff MR, Dodin A (1985) *J Clin Microbiol* **22**: 873–6.
7 Corfield T (1992) *Glycobiology* **2**: 509–21.
8 Müller HE (1992) *BiOforum* **1–2**: 16–22.
9 Roggentin P, Schauer R, Hoyer LL, Vimr ER (1993) *Mol Microbiol* **9**: 915–21.
10 Roggentin T, Kleineidam RG, Schauer R, Roggentin P (1992) *Glyconconj J* **9**: 235–40.
11 Roggentin P, Kleineidam RG, Schauer R (1995) *Biol Chem Hoppe-Seyler* **376**: 569–75.
12 Messing J (1983) *Methods Enzymol* **101**: 20–78.
13 Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) *Methods Enzymol* **185**: 60–89.
14 Yannisch-Perron C, Vieira J, Messing J (1985) *Gene* **33**: 103–19.
15 Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Second Edition. Cold Spring Harbor: Laboratory Press.
16 Roggentin P, Rothe B, Lottspeich F, Schauer R (1988) *FEBS Lett* **238**: 31–4.
17 Kruse S, Kleineidam RG, Roggentin P, Schauer R (1996) *Protein Expression and Purification* **7**: 415–22.
18 Laemmli UK (1970) *Nature* **227**: 680–5.
19 Potier M, Mameli L, Bélisle M, Dallaire L, Melançon SB (1975) *Anal Biochem* **94**: 287–96.
20 Ellis KJ, Morrison JF (1982) *Methods Enzymol* **87**: 405–26.
21 Veh RM, Michalski JC, Corfield AP, Sander M, Schauer R (1981) *J Chromat* **212**: 313–22.
22 Reuter G, Schauer R (1994) *Methods Enzymol* **230**: 168–97.
23 Ray WJ, Koshland DE (1961) *J Biol Chem* **236**: 1973–9.
24 Siebert HC, Tajkhorshid E, von der Lieth CW, Kleineidam RG, Kruse S, Schauer R, Kaptein R, Gabius HJ, Vliegenhart JFG (1996). *J Mol Model* **2**: 446–55.
25 Crennell SJ, Garman EF, Laver WG, Vimr ER, Taylor GL (1993) *Proc Natl Acad Sci USA* **90**: 9852–6.
26 Crennell SJ, Garman EF, Philippon C, Vasella A, Laver WG, Vimr ER, Taylor GL. (1996) *J Mol Biol* **259**: 264–80.
27 Heuermann D, Roggentin P, Kleineidam RG, Schauer R (1991) *Glycoconj J* **8**: 95–101.

28 Traving C, Schauer R, Roggentin P (1994) *Glycoconj J* **11**: 141–51.
29 Rothe B, Roggentin P, Frank R, Blöcker H, Schauer R (1989) *J Gen Microbiol* **135**: 3087–96.
30 Akimoto S, Ono T, Tsutsui H, Kinouchi T, Kataok a K, Ohnishi Y (1994) *Biochem Biophys Res Commun* **203**: 914–21.
31 Rothe B, Rothe B, Roggentin P, Schauer R (1991) *Mol Gen Genet* **226**: 190–7.
32 Thompson JD, Higgins DG, Gibson TJ (1994) *Nucleic Acids Res* **22**: 4673–80.

Received 12 November 1997, revised 23 December 1997, accepted 2 March 1998