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Effects of site-specific mutations on the enzymatic properties of a sialidase from *Clostridium perfringens*

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Three site-specific mutations were performed in two regions of a sialidase gene from *Clostridium perfringens* which are known to be conserved in bacterial sialidases. The mutant enzymes were expressed in *Escherichia coli* and, when measured with MU-Neu5Ac as substrate, exhibited variations in enzymatic properties compared with the wild-type enzyme. The conservative substitution of Arg 37 by Lys, located in a short conserved region upstream from the four repeated sequences common in bacterial sialidase genes, was of special interest, as K_M and V_{max}, as well as K_i measured with Neu5Ac2en, were dramatically changed. These data suggest that this residue may be involved in substrate binding. In addition to its low activity, this mutant enzyme has a lower temperature optimum and is active over a more limited pH range. This mutation also prevents the binding of an antibody able to inhibit the wild-type sialidase. The other mutations, located in one of the consensus sequences, were of lower influence on enzyme activity and recognition by antibodies.

Keywords: sialidase (neuraminidase), site-specific mutations, conserved sequences, enzymatic properties, *Clostridium perfringens*

Sialidases (neuraminidases, EC 3.2.1.18) catalyse the release of sialic acids from complex carbohydrates [1]. They are essential in metazoan animals of the deuterostomate lineage for the turnover of sialoglycoconjugates. Some pathogenic microorganisms such as protozoa, bacteria and viruses produce sialidases for nutritional reasons or to facilitate interaction with their animal hosts [2–4].

On a molecular level, the sialidases of influenza viruses type A and B are well characterized (for a review see [5]). The primary and the 3-dimensional structures of a number of virus sialidases have been determined [5–11]. For the catalytic mechanism of influenza A virus sialidase a hypothesis has been published [12]. A histidine and a glutamic acid residue were postulated to be involved in catalysis [12]. Together with other amino acids, they line the large pocket on the viral protein surface, which binds the sialic acid product [8]. The modification of conserved arginine, tryptophan, threonine and aspartic acid residues had a considerable influence on enzyme activity of virus sialidases [12, 13], but their participation in substrate binding or catalysis had not yet been proved unequivocally. Recently, Burmeister *et al.* [11] published a refined crystal structure of influenza B virus sialidase with bound *N*-acetylneuraminic acid exhibiting a differently positioned active site. They emphasize the importance of arginine residues amongst other amino acids for sialic acid binding.

The genes of clostridial and other bacterial sialidases have no significant sequence homologies to influenza virus sialidases [14, 15]. A comparison of the predicted amino acid sequences of all published bacterial sialidase genes gave no indication of conserved histidine or glutamic acid residues (unpublished results). Nevertheless, the mechanism of substrate binding and catalysis could be the same. Virus genes undergo very fast evolution. Therefore, an original homology with bacterial sialidases may have disappeared or no longer be perceptible.

Comparison of the primary structures of bacterial sialidases resulted in the discovery of a short stretch of twelve amino acids which, in most cases, is repeated at four positions in these proteins. Five of these amino acids are highly conserved [14]. Strikingly, the respective distances between the repeated domains in different bacterial sialidases are often similar [14]. A short sequence of four amino acids about 37 positions upstream of the first repeated region was also found in all bacterial sialidases [15].

These conserved regions contain amino acids possibly involved in enzyme function, as indicated by chemical modification experiments with other sialidases. Bachmeyer [16] described tryptophan to be essential for the activity of the secreted sialidase from *Clostridium perfringens*; Kabayo

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and Hutchinson [17] inactivated a sialidase from *Streptomyces griseus* by the modification of arginine.

In the present study with the small sialidase of *C. perfringens* [18], three amino acids, two of which are highly conserved when compared with other bacterial sialidases, were substituted by other amino acids using direct-site mutagenesis. The mutant enzymes were characterized in order to gain insight into the function of the conserved domains in this and other bacterial sialidases.

Materials and methods

Materials

Restriction endonucleases, ligase and other DNA manipulating enzymes were purchases from Gibco (Eggenstein, Germany). λ-DNA and the M13 sequencing kit were obtained from Boehringer (Mannheim, Germany). The molecular weight marker was prepared by restriction of λ-DNA with Hind III and Eco R I. Vectors pMac5-8, *E. coli* strains WK6 and WK6mutS, and the M13K07 helper phage were part of a mutagenesis system which was a gift from H. J. Fritz (Göttingen, Germany). γ-[^{32}P]ATP, α-[^{35}S]ATP and blotting membranes (Hybond N) were from Amersham (Braunschweig, Germany). Oligonucleotides were synthesized by R. Söller (Kiel, Germany) with a DNA synthesizer (380 B, Applied Biosystems, Pfungstadt, Germany). Antibodies against the pure wild-type sialidase and the 'large' isoenzyme of *C. perfringens* were raised in rabbits and sheep, respectively, and were prepared as described [19]. 4-Methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac) was synthesized and purified as described earlier [20]. All other chemicals were of analytical grade and purchased from Boehringer, Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) or Serva (Munich, Germany).

Oligonucleotide-directed site-specific mutagenesis

All common DNA manipulations, like cloning, transformation, DNA-labelling, hybridizations and agarose gel-electrophoresis, were carried out according to standard protocols [21]. The mutagenesis of the sialidase gene of *C. perfringens* strain A99 [18] was performed with the vector system described by Stanssens *et al.* [22]. A 1.8 kb Hind III fragment of strain A99 DNA encoding the complete sialidase gene was subcloned in the vector pMa5-8 and propagated in *E. coli* WK6. Clones expressing sialidase activity were identified by spraying the colonies with 1 mM MU-Neu5Ac in distilled water. Positive clones were visualized by their fluorescence under UV light at 354 nm. Mutagenesis with oligonucleotides of 27 bases length was performed according to the procedure of Stanssens *et al.* [22] with one modification: mutants were identified by colony blotting and hybridization with the respective γ-[^{32}P]ATP labelled mutagenic oligonucleotide [21]. The molecular weight and amount of phasmids encoding the mutated gene were checked by restriction of quantitative phasmid preparations with Hind III, followed by agarose gel electrophoresis and comparison with the λ standard. In order to confirm each correct mutation on the sialidase gene, the phasmid was generated in single stranded template [22] and the surrounding 200 nucleotides were sequenced with an M13 sequencing kit (Boehringer) using oligonucleotide primers designed from the known sequence.

Enzyme preparation

After 1:100 inoculation, recombinant *E. coli* cells were grown for 16 h at 37 °C in Luria broth [21] supplemented with 25 μg ml^{-1} chloramphenicol. After harvesting (10 min, 10 000 × g, 4 °C), the cells were resuspended in 1/10 culture volume of 25 mM Tris-HCl, pH 8.0, containing 50 mM glucose, 10 mM EDTA and 10 mg ml^{-1} lysozyme (lysozyme buffer). After incubation for 30 min at 37 °C, the sialidase protein was found in the supernatant after centrifugation. The amount of this protein was quantified by densitometric measurement (Densitometer CD 60, Desaga, Heidelberg, Germany) of the respective band obtained by separation of supernatant proteins by SDS-polyacrylamide gel electrophoresis (12%T, 3.75%C, 6 h, 40–60 V) and silver-staining [23, 24]. Purified (unpublished) wild-type sialidase served as standard.

Sialidase activity was routinely determined with the fluorogenic substrate MU-Neu5Ac as described by Potier *et al.* [25]. Unless stated otherwise, each assay contained 80 μl 0.1 M sodium acetate buffer, pH 6.1, 10 μl sialidase solution and 10 μl 1 mM MU-Neu5Ac. The reaction was terminated after 10 min at 37 °C by addition of 0.9 ml 0.133 M glycine buffer, pH 10, containing 60 mM NaCl and 40 mM Na_2CO_3. The fluorescence of the released 4-methylumbelliferone (MU) was measured in a Perkin-Elmer (M-1000) filter-spectrofluorimeter using excitation at 365 nm and emission at 450 nm. The fluorimeter was calibrated with MU standard solutions.

Enzyme characterization

All studies on enzyme properties of the wild-type and the mutant sialidases were performed with lysozyme supernatants and MU-Neu5Ac as substrate. If necessary, supernatants with high sialidase activity were diluted with lysozyme supernatant from *E. coli* cells bearing pMc5-8 without insert, which was prepared as described above. One unit (U) of enzyme activity was defined as 1 μmol substrate hydrolysed per minute.

The influence of pH on enzyme activity was measured by applying three different buffer systems in 0.5 pH steps: (i) 0.143 M sodium acetate buffer (pH range 3–7); (ii) 0.143 M sodium phosphate buffer (pH range 5–9); (iii) a three buffer mixture with constant ionic strength [26], containing 0.1 M acetic acid, 0.1 M 2-(*N*-morpholino)ethanesulfonic acid

(MES) and 0.2 M triethanolamine, the pH of which was adjusted with HCl or tetramethylammonium hydroxide (pH range 3–9). From acetate or phosphate buffer 70 µl were used in each test, resulting in a final concentration of the buffer anion of 0.1 M. After the addition of 20 µl sialidase solution, the assay was started with 10 µl MU-Neu5Ac and stopped after 10 min at 37 °C. The assay employing the third buffer contained 50 µl buffer (concentration of each buffer component in the test mixture was 0.05 M and the ionic strength 0.1), 20 µl water, 20 µl sialidase solution and 10 µl 1 mM MU-Neu5Ac, and was performed at 30 °C for 10 min, and additionally for 40 min in the case of the K3 enzyme. The sialidases were diluted to an activity of about 10 mU ml^{-1} in the standard assay, to be in the linear range of the fluorimetric measurement.

The temperature dependence of enzyme activity was determined in the standard assay with sialidases diluted to about 20 mU ml^{-1} at 37 °C. Temperatures from 0 °C to 55 °C were tested in steps of 5 °C. Each sample in an Eppendorf tube was prewarmed for 2 min to reach the respective temperature, before the reaction was started with 10 µl 1 mM MU-Neu5Ac solution. Values presented are the average of at least three tests.

The stability of the sialidases was measured at 37 °C in lysozyme buffer (for composition see above). After 5, 10, 20, 40, 80 and 160 min, 10 µl aliquots were assayed under standard conditions.

The linearity of the hydrolytic activity of each sialidase was followed over 2 h in assays containing 70 µl 0.143 M sodium acetate buffer, pH 6.1, 20 µl sialidase solution of about 1 mU ml^{-1} and 10 µl 1 mM substrate solution. Free MU was determined after 10, 30, 60, 90 or 120 min of incubation at 37 °C.

For the determination of K_M-values, MU-Neu5Ac was hydrolysed at final concentrations of 1, 0.5, 0.2, 0.1 and 0.06 mM under standard conditions with the sialidase solutions adjusted to about 20 mU ml^{-1}. Inhibition by 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) was investigated at 0.01, 0.05, 0.25 mM, and with the K3-mutant enzyme also at 1 mM using the substrate concentrations given above. Each measurement was performed in triplicate. K_M and V were calculated by fitting the kinetic data to the Michaelis–Menten equation by nonlinear regression with the program Enzfitter (Elsevier Biosoft, Oxford, UK). Inhibition data were fitted with the nonlinear regression program of Duggleby [27] to the variants of the Michaelis–Menten equation for competitive, noncompetitive, uncompetitive, and mixed inhibition.

For antibody inhibition tests, sialidase solutions obtained from wild-type and clones were diluted to activities between 18 and 267 µU per 20 µl with lysozyme supernatants as described above. The tests were performed by mixing 20 µl antibody solution (2.5 mg dry, desalted antibody powder dissolved in 1 ml 0.1 M Tris-HCl buffer, pH 7.4, supplemented with 1% (w/v) bovine serum albumin) with 20 µl sialidase solution, followed by 30 min incubation at 37 °C before substrate was added. In parallel, 20 µl sialidase solution was mixed with 20 µl of antibody dilution buffer and treated as the inhibition sample. After preincubation, 50 µl 0.1 M sodium acetate buffer, pH 6.1, and 10 µl 1 mM MU-Neu5Ac were added to each sample. Hydrolysis was stopped after 10 or 30 min at 37 °C and activity was estimated as described above. Inhibition values were calculated from the differences in activity between samples with or without antibodies added. The K3 sialidase was additionally incubated with antibodies raised against the 'large' sialidase isoenzyme of *C. perfringens* [19] under identical conditions. All values presented are the average of at least three estimations.

Results and discussion

Based on the considerations outlined in the Introduction section, three mutations were performed in the conserved regions of the sialidase gene from *C. perfringens*:

K1 (Asp 143 → Lys)

K2 (Trp 149 → Ala)

K3 (Arg 37 → Lys)

Their location in the amino acid sequence of the sialidase [18] is shown in Fig. 1. The mutations K1 and K2 are located in the second repeated region, while the K3 amino acid is part of a short conserved sequence upstream from the repeated domains. Asp 143 (K1) does not belong to the highly conserved amino acids, as in the third repeated domain a lysine residue is present at this position. This mutation, however, was selected in order to introduce a minor disturbance, from which an active sialidase was expected as a control for the mutagenesis system. Trp 149 (K2) and Arg 37 (K3) are highly conserved amino acids. The change of Trp 149 to Ala removed the aromatic moiety and thereby its ability to form charge-transfer interactions and hydrogen bonds. Arg 37 (K3) was replaced by lysine, which was selected on the basis of its similarity to Arg, thereby minimizing any influence on the tertiary structure of the enzyme.

Wild-type and mutant genes were expressed in *E. coli*. As the phasmid pMac5-8 is not an expression vector, it must be assumed that the sialidase gene was expressed from its own promoter. The nonsecreted wild-type sialidase is a soluble monomer with a molecular weight of 41 000 as determined with SDS-polyacrylamide gel electrophoresis (unpublished results). A comparison of the wild-type and mutant enzymes was carried out with lysozyme supernatants, as the wild-type enzyme is very sensitive to some purification procedures, especially dilutions, and it is unlikely that the mutant enzymes exhibit higher stability during this procedure. The lysozyme supernatants contained almost identical relative amounts of the respective proteins,

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+1   M C N K N N T F E K N L D I S H K P E P L I L F N K D N N I W N S K Y F[R]I P N +40
+41  I Q L L N D G T I L T F S D I R Y N G P D D H A T I D I A S A R S T D F G K T W +80
+81  S Y N I A M K N N R I D S T Y S R V M D S T T V I T N T G R I I L I A G S W N T +120
+121 N G N W A M T T S T R R S D W S V Q M I Y S[D]D N G L T[W]S N F I D L T K D S S +160
+161 K V K N Q P S N T I G W L G G V G S G I V M D D G T I V M P A Q I S L R E N N E +200
+201 N N Y Y S L I I Y S K D N G E T W T M G N K V P N S N T S E N M V I E L D G A L +240
+241 I M S T R Y D Y S G Y R A A Y I S H D L G T T W E I Y E P L N G L I L T G K G S +280
+281 G C Q G S F I K A T T S N G H R I G L I S A P K N T K G G Y I R D N I A V Y M I +320
+321 D F D D L S K G V Q E I C I P Y P E D G N K L G G G Y S C L S F K N N H L G I V +360
+361 Y E A N G N I E Y Q D L T D Y Y S L I N K Q +382
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Figure 1. Primary structure of the 43000 *Clostridium perfringens* sialidase deduced from the nucleotide sequence [18]. Highly conserved sequences, e.g., the repeated sequences, are underlined. The localization of the mutations is framed.

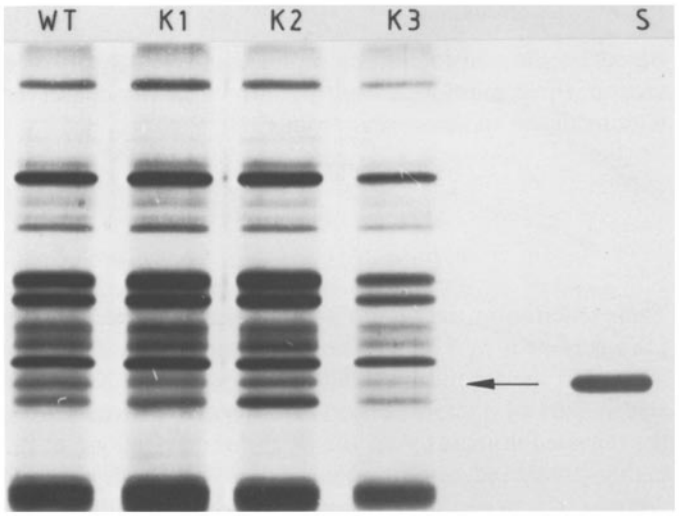

Figure 2. Silver-stained protein bands of equal amounts of lysozyme supernatants, which were derived from *E. coli* clones expressing wild-type (WT) and mutant (K1, K2, K3) enzymes, separated by SDS-PAGE. Lane S exhibits one band of pure WT sialidase, indicating the position of the cloned proteins (arrow).

which was also valid for the sialidase protein, as shown in Fig. 2. Since the sialidase protein was completely separated on the gels from other proteins, it was possible to compare the amounts of mutant enzymes with that of the wild-type.

All three mutant enzymes exhibited sialidase activity as measured with MU-Neu5Ac. This synthetic substrate permits the quick and reliable determination of even very low sialidase activities (>0.1 mU ml^{-1}), which is not possible with natural substrates. Furthermore, this sialidase assay is not affected by other sugars or by the product degrading sialate lyase (EC 4.1.3.3), which may generate problems with assays determining the liberated sialic acids.

The time-dependent hydrolysis of MU-Neu5Ac by all four enzymes was constant for at least 30 min under standard conditions, i.e., 37 °C and pH 6.1. WT, K1, and K2 exhibited a decrease in activity thereafter, while the hydrolysis rate of the K3 enzyme was constant up to 120 min (data not shown). The loss of linearity of hydrolysis by WT, K1, and K2 was not caused by lability of the enzymes, as preincubation under identical conditions followed by determination of enzyme activity showed no decrease in turnover. Moreover, inhibition by the reaction products *N*-acetylneuraminic acid and methylumbelliferone can be excluded, since no inhibitory effect at concentrations of up to 20 μM was observed.

The kinetic properties of the mutant sialidases vary considerably, as shown in Table 1: K1 shows a behaviour similar to the wild-type, as is reflected in very similar K_M and V, as well as K_i, the inhibitor constant for competitive inhibition by Neu5Ac2en. The maximum velocity of K2 was reduced to 17% of that of the wild-type enzyme, whereas K_M and K_i were similar. K3 exhibited only a very low V (1.2% of WT), and its K_M value is about ten times higher than that of WT, suggesting impaired substrate binding. This is also indicated by the fact that Neu5Ac2en, which is a competitive inhibitor of WT, K1, and K2 (K_i values between 0.16 and 0.24 mM), is an uncompetitive and weak inhibitor for K3 sialidase with a K_i value of 4.3 mM. The temperature dependence of enzyme activities is shown in Fig. 3. The behaviour of WT, K1 and K2 is nearly identical, exhibiting an optimum at about 40 °C. However, at

Table 1. Michaelis constants K_M, maximal velocities V, and competitive inhibitor constants K_i of wild-type and mutant sialidases determined with MU-Neu5Ac and Neu5Ac2en.

	K_M (mM)[a]	V (U ml^{-1})[b]	K_i (mM)
WT	0.19	6.9	0.16
K1	0.17	7.2	0.24
K2	0.21	1.2	0.17
K3	1.8	0.081	4.3[c]

[a] K_M values are the mean of two independent determinations with deviations of less than 25%.
[b] V is expressed as activity per ml lysozyme supernatant and is corrected for the different sialidase concentrations.
[c] Uncompetitive inhibition.

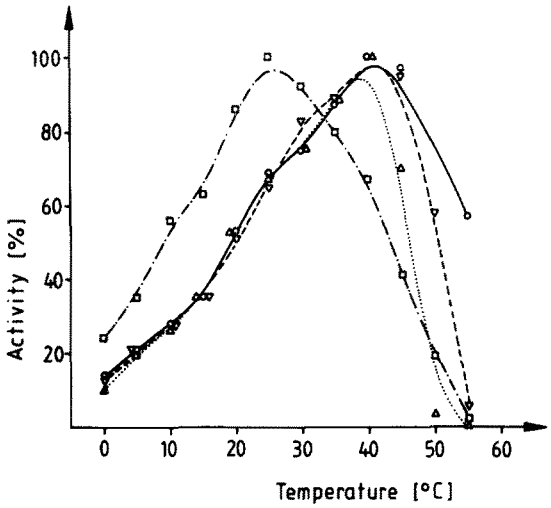

Figure 3. Temperature dependence of the activities of wild-type (○) and mutant (K1 ▽; K2 △; K3 □) sialidases measured in 0.1 M sodium acetate buffer, pH 6.1.

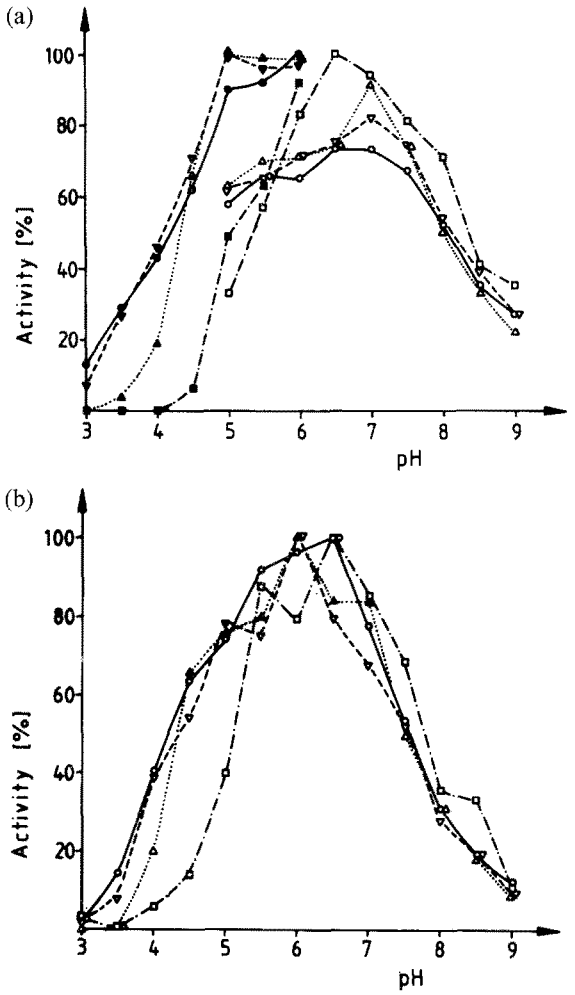

Figure 4. Dependence on pH of the activities of the sialidases WT (●, ○), K1 (▼, ▽), K2 (▲, △), and K3 (■, □) measured with (a) acetate buffer (●, ▼, ▲, ■) and phosphate buffer (○, ▽, △, □), and (b) with the three-buffer system (see the Materials and methods section).

temperatures above 45 °C, the mutant enzymes are less active than the WT, indicating some minor disturbances of their stability. The temperature curve of K3 is nearly bell-shaped, with an optimum at 25 °C, showing a less steep decline at higher temperatures than the other enzymes. Taking into account the enzyme's stable activity at 37 °C mentioned above, this comparatively slow decline seems not to be an effect of denaturation, but possibly of a second stable state with lower activity.

The pH curves of the sialidases are presented in Fig. 4. Similar to the properties described so far, the behaviour of K1 and K2 corresponds to that of the WT enzyme showing a broad pH optimum from pH 4.5 to pH 7.0. The activity of K2 is slightly lower at pH values below 4. The K3 enzyme is inactive below pH 4 and has its optimum at pH 6.5 in the buffer systems tested. The lower activities of WT, K1, and K2 in 0.1 M phosphate compared with the activities in 0.1 M acetate buffer are probably caused by the higher ionic strength of the former system. Comparable ionic strength effects have already been described as a cause of inhibition of the sialidases of *Clostridium sordellii* [28] and of *Clostridium chauvoei* [29]. This effect is much reduced with the K3 enzyme. The higher relative activities of WT and K1 in the pH range between 3.0 and 4.0 in the 0.1 M acetate buffer, when compared with the three buffer system with an ionic strength of 0.1, were probably also caused by the lower ionic strength of the former buffer.

Antibodies raised against WT sialidase inhibited the activity of the mutant enzymes of K1 and K2 to a degree comparable with the homologous enzyme. Remarkably, the K3 sialidase was not inhibited, but even slightly activated by these antibodies, as is demonstrated in Table 2. These observations indicate that Arg 37 is an essential part of an antigenic site on the wild-type protein which, after binding of the respective antibody, is responsible for inhibition of the WT enzyme. Furthermore, only one antigenic site seems

Table 2. Comparison of the effect of antibodies (AB) raised against the pure WT sialidase on wild-type (WT) and mutant (K1, K2, K3) sialidases at two different concentrations. For the sialidase antibody inhibition assay see the Materials and methods section.

Sialidase	*Sialidase activity*		*Residual activity (%)*
	−AB[a]	*+AB*	
WT	135	12	9
	18	1	6
K1	167	13	8
	34	2	6
K2	267	39	15
	29	2	7
K3	121	158	131
	22	26	118

[a] Sialidase activity (μU per 20 μl) set at 100% for calculation of residual activity.

to be present, which enables inactivation after antibody binding since, after change of Arg 37 to Lys, inactivation was no longer detectable with this assay. The slight increase in activity of K3 enzyme after antibody treatment cannot be explained, but it probably is not an unspecific effect, as the heterologous 'large' sialidase antibodies had no influence on K3 sialidase activity.

As all mutant forms of the sialidase of *C. perfringens* described here show enzyme activity, the folding of the mutant proteins seems not to be significantly altered. The K1 enzyme exhibits essentially the same properties as the wild-type form, indicating that the type of amino acid at this less conserved position in a well conserved region is of minor importance for function. K2 exhibited a reduced activity, but this effect is less than that predicted from the inactivation by chemical modification of tryptophan [16]. It remains to be tested whether the mutation of tryptophan at one of the other three conserved and repeated regions might result in more pronounced effects on enzyme function or structure.

The K3 mutation obviously has the greatest effect on catalysis. The alteration of all tested properties of this sialidase complicates the interpretation of the role of arginine 37. The strong increase in the K_M value of the mutant enzyme and the inability of Neu5Ac2en to inhibit it competitively, in contrast to the wild-type enzyme, to the other mutants and to most sialidases from other origins [1] indicate the participation of arginine 37 in substrate binding. This role may be analogous to the importance of arginine residues for sialic acid binding by influenza B virus sialidase [11].

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