Crystal structure of a bacterial sialidase (from Salmonella typhimurium LT2) shows the same fold as an influenza virus neuraminidase

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ABSTRACT Sialidases (EC 3.2.1.18 or neuraminidases) remove sialic acid from sialoglycoconjugates, are widely distributed in nature, and have been implicated in the pathogenesis of many diseases. The three-dimensional structure of influenza virus sialidase is known, and we now report the three-dimensional structure of a bacterial sialidase, from Salmonella typhimurium LT2, at 2.0-Å resolution and the structure of its complex with the inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid at 2.2-Å resolution. The viral enzyme is a tetramer; the bacterial enzyme, a monomer. Although the monomers are of similar size (~380 residues), the sequence similarity is low (~15%). The viral enzyme contains at least eight disulfide bridges, conserved in all strains, and binds Ca2+, which enhances activity; the bacterial enzyme contains one disulfide and does not bind Ca2+. Comparison of the two structures shows a remarkable similarity both in the general fold and in the spatial arrangement of the catalytic residues. However, an rms fit of 3.1 Å between 264 Ca atoms of the S. typhimurium enzyme and those from an influenza A virus reflects some major differences in the fold. In common with the viral enzyme, the bacterial enzyme active site consists of an arginine triad, a hydrophobic pocket, and a key tyrosine and glutamic acid, but differences in the interactions with the O4 and glycerol groups of the inhibitor reflect differing kinetics and substrate preferences of the two enzymes. The repeating "Asp-box" motifs observed among the nonviral sialidase sequences occur at topologically equivalent positions on the outside of the structure. Implications of the structure for the catalytic mechanism, evolution, and secretion of the enzyme are discussed.

Sialidase was originally identified as a "receptor-destroying enzyme" in extracts of Vibrio cholerae because of its ability to release influenza virus from the surface of erythrocytes. Sialidases have been found in viruses, bacteria, trypanosomes, and mammalian cells (1, 2). There is evidence for two families of the bacterial enzyme, distinguished by a requirement for a divalent metal ion for maximal activity. Those not requiring metal have molecular weights of ~42,000 and share some sequence similarity—e.g., the sialidases of Clostridium perfringens, Clostridium sordelli, Salmonella typhimurium, and Micromonospora viridifaciens. These enzymes also share sequence similarity with the N-terminal domain of the membrane-bound sialidase of Trypanosoma cruzi (3). However, the sialidase from V. cholerae, which has been cloned (4), sequenced (5), and crystallized (6), has a molecular weight of 82,000 and requires a metal ion. The influenza virus enzymes possess a calcium-binding site, and although calcium is not essential for activity, it does enhance activity (7). Within the nonviral enzymes there is evidence for a conserved sequence motif (Ser/Thr-Xaa-Asp-[Xaa]-Gly-Xaa-Thr-Trp/Phe), or "Asp-box," which repeats three to five times along the sequences (8).

Comparison of Bacterial and Viral Structures

The only sialidase three-dimensional (3-D) structure previously known was that of the influenza virus enzyme (9–11). The influenza virus sialidase forms tetramers on the virus surface, which remain as tetramers when released from the virus by Pronase, whereas other sialidases investigated to date may be monomers (12). The viral monomer has a molecular weight similar to the small sialidases, requires a divalent metal ion for maximal activity, and does not generally possess the Asp-box motifs.

We have determined the crystal structure of a sialidase from S. typhimurium by the method of multiple isomorphous replacement (MIR). The structure has been refined to 2.0-Å resolution with a crystallographic R value of 0.189. The statistics are presented in Table 1, and the experimental details are given in its legend.

A schematic view of the enzyme is shown in Fig. 1. The enzyme is mainly β-sheet with two small α-helical segments, with a shallow active site crevice, identified crystallographically by the soaking of DANA into crystals (Fig. 3), and on the opposite side it has a deep cleft extending ~15 Å into the structure. This cleft proved effective in allowing differential binding of two Hg derivatives used in the phasing; pCMB with its bulky aromatic group was unable to penetrate the cleft, whereas HgCl2 was able to reach cysteines deep inside the cleft. The fold topology is identical to that found in the influenza virus sialidases and consists of six four-stranded antiparallel β-sheets arranged as the blades of a propeller around an axis passing through the active site. However, there are major differences in the lengths of the β-strands and the loops between the sheets.

Unlike the S. typhimurium structure, the viral enzyme has a C-terminal extension which is involved in maintaining the tetramer through interactions with the first and second sheets of adjacent monomers. A sequence alignment based on structures of the bacterial enzyme and a viral enzyme is given in Fig. 2. In all influenza A and B sialidases sequenced to date, there are eight totally conserved disulfide bridges, which brace the sheets (25). In the S. typhimurium structure,

Abbreviations: 3-D, three-dimensional; MIR, multiple isomorphous replacement; pCMB, p-chloromercuribenzoate; DANA, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid; NANA, N-acetylneuraminic acid.

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†The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (entry codes 1SIL and 1SIM).
Table 1. X-ray data collection and phasing statistics

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<th>Native</th>
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<td>2 (A, B)</td>
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\[ R_I = \sum I - \langle I \rangle/\langle I \rangle; \] phasing power = rms \( f_u \)/lack of closure; Cryst refinement is reported in phosphate geometry.

Comparison of the Active Sites

The catalytic site of the influenza A and B virus sialidases has been characterized by binding of the inhibitor DANA or of the product N-acetylneuraminic acid (NANA), which is a weak inhibitor of the viral enzyme (11, 26, 28). Kinetic studies on the \( S. typhimurium \) enzyme have shown inhibition by DANA with a \( K_i \) similar to the viral enzyme but no inhibition by NANA, and a kinetic preference for sialyl \( \alpha_2 \rightarrow \alpha_3 \) linkages over \( \alpha_2 \rightarrow \alpha_6 \) similar to the influenza virus (29). The catalytic site shares several features with the viral enzyme (Fig. 4): (i) Three arginines residues (37, 246, and 309) stabilize the carboxylic acid group common to all natural sialic acid derivatives. (ii) A glutamic acid (361) stabilizes the position of one of the triad arginines. (iii) A tyrosine (342) approaches the sugar ring of the sialic acid from below; the tyrosine-hydroxyl group is only 3.0 Å from the C1 and C2 carbons of DANA, a fact that lends support to its role in stabilizing a carbonium ion transition state intermediate (30). (iv) A hydrophobic pocket which accommodates the \( N \)-acyetyl group of sialic acid is formed by Met-99, Trp-121, Trp-128, and Leu-175; in the viral enzyme it is formed by a tryptophan and an isoleucine. (v) A glutamate (231) donates a proton, a step that has been implicated in the viral enzyme mechanism (30).

Significant differences between the bacterial and viral active sites are as follows: (i) Interactions with sialic acid glycerol group. In the viral enzyme a glutamic residue forms two strong hydrogen bonds (2.5–2.9 Å) with O8 and O9 of the glycerol side group of DANA and NANA (11, 28), while the \( S. typhimurium \) enzyme has only one direct weak hydrogen bond, from Trp-128 to O9. The large difference in turnover number of 2700 s\(^{-1}\) for the \( S. typhimurium \) enzyme (29) and \( \approx 10 \) s\(^{-1}\) for the viral enzyme (G. Air, personal communication) could be explained by the extra stabilization of the glycerol in the case of the viral enzyme, as it is not uncommon for product release to be the limiting factor in catalysis (31). This difference could also explain why a derivative of DANA with a bulky arylazide group at O9, in place of the hydroxyl, is bound by the bacterial enzymes (32). (ii) Interactions with sialic acid N4. Uniquely, the \( S. typhimurium \) enzyme has Asp-100 and Arg-56 making strong hydrogen bonds to the O4 of DANA (2.8 and 2.9 Å, respectively), plus a weak hydrogen bond from Asp-62 (3.2 Å); the viral enzyme just has the latter weak hydrogen bond from its corresponding asparatic residue. This difference explains the observation that N-acetyl-4-O-acetyleneuraminyl-(2→3)-lactose is hydrolyzed by the viral enzyme but not by a bacterial enzyme (33): the bulky acetyl group inhibits binding to the bacterial enzyme, whereas in the viral enzyme there is room to accommodate this extra group, and substitution at this site has produced the most effective viral enzyme inhibitors to date (34).

Discussion

A recent comparison of seven sialidase sequences from five different genera and two protozoan sialidase sequences from \( T. cruzi \) demonstrates homology (common descent) among small and large enzymes, consistent with the existence of a sialidase superfamly (12, 20). Together with the current structural analysis of the \( S. typhimurium \) sialidase active site, the results indicate that all sialidases should have similar catalytic mechanisms. Our current results strongly indicate that the influenza virus enzyme is part of the sialidase superfamly. Furthermore, the possibility that bacteria originally acquired the sialidase genetic information from eukaryotes is consistent with the phylogenetic distribution of sialic acids and sialidases (12, 20) and with the structural similar-
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S. typhimurium sialidase with DANA

Influenza virus neuraminidase (Tern N9)


FIG. 1. Orthogonal views of the sialidase from S. typhimurium with bound inhibitor DANA (Upper) and the neuraminidase from tern influenza virus subtype N9 (Lower) produced by the MOLSCRIPT program (23), looking from the side (Left) and from above the active site (Right). The N9 structure is viewed after optimizing its fit to the bacterial structure by using the program sftp (24), which gave an rms fit of 3.1 Å for 264 Cα atoms. In both structures, the chain is colored from red at the N terminus to violet at the C terminus. (The sequences of the enzymes are aligned in Fig. 2.) Note that, in both enzymes, the sixth sheet is formed from an N-terminal β-strand and three C-terminal β-strands. Note that in the Lower Right view the viral tetramer is formed by a fourfold axis approximately through the bottom right of the picture and normal to the page.

The function(s) of bacterial sialidases is (are) not entirely clear. Nucleotide sequencing and biochemical analysis of S. typhimurium nanH and its encoded sialidase indicated a primarily intracellular enzyme location and the absence of a typical prokaryotic signal sequence (21, 29). When this nanH copy was subcloned in a sialidase-negative V. cholerae strain, which normally excretes its own sialidase (4), up to 25% of the total S. typhimurium sialidase activity was found in the culture medium compared with less than 2% of β-lactamase encoded by the vector (S. M. Steenbergen and E.R.V., unpublished data). It may be that the Asp boxes, which are not generally conserved in the viral sialidases, are involved with sialidase secretion and that these "signals" are not being efficiently recognized by S. typhimurium. Regardless of whether Asp boxes play any role in secretion, S. typhimurium LT2 is able to use sialyl-a2-3-lactose as a sole carbon and energy source, while a nanH mutant strain cannot (S. M. Steenbergen and E.R.V., unpublished data), consistent with a primarily nutritional function for most bacterial sialidases (20). Thus, any role of the enzyme in bacterial
pathogenesis may be a secondary consequence of this nutritional function. Knowledge of bacterial sialidase structure-function will be central to the design of compounds for the treatment and prevention of diseases caused by sialidase-positive microbes, and it should help to provide insight into a variety of noninfectious diseases of humans that involve derangements in sialic acid catabolism.

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Fig. 4. Stereoviews of the active site. (Upper) S. typhimurium sialidase with DANA. (Lower) Tern influenza virus N9 sialidase with DANA (26). Hydrogen bonds (<3.2 Å) are drawn as broken lines. Water molecules (H2O) in Upper are drawn as two concentric circles. Both sites are viewed from the same direction, looking into the active site. The N9 structure has been optimally fitted to the bacterial structure as described in the legend of Fig. 1. The Cα coordinates of seven residues common to both sites have an rms fit of 1.01 Å (Arg-37/118, Asp-62/151, Glu-231/277, Arg-246/292, Arg-309/371, Tyr-342/406, and Glu-361/425).