Sialic acid presentation on the cell surface by some patho-
genic strains of bacteria allows their escape from the host
immune system. It is one of the major virulence factors. Bacte-
rial biosynthesis of sialic acids starts with the conversion of
UDP-GlcNAc to UDP and ManNAc by a hydrolyzing 2-epi-
merase. Here, we present the crystal structure of this enzyme,
named NeuC, from Acinetobacter baumannii. The protein folds
into two Rossmann-like domains and forms dimers and tetra-
mers as does the epimerase part of the bifunctional UDP-GlcNAc
2-epimerase/ManNAc kinase (GNE). In contrast to human
GNE, which showed only the closed conformation, the NeuC
crystals contained both open and closed protomers in each
dimer. Substrate soaking changed the space group from C2221
to P2_1212_1. In addition to UDP, an intermediate-like ligand was
seen bound to the closed protomer. The UDP-binding mode in
NeuC was similar to that in GNE, although a few side chains
were rotated away. NeuC lacks the CMP-Neu5Ac-binding site
for allosteric inhibition of GNE. However, the two enzymes as
well as other NeuC homologues (but not SiaA from Neisseria
meningitidis) appear to be common in tetrameric organization.
The revised two-base catalytic mechanism may involve His–125
(Glu-134 in GNE), as suggested by mutant activity analysis.

Sialic acids are a family of nine-carbon α-keto sugars with
1-carboxylate group. More than 50 different species of sialic
acids are found in nature, among which N-acetylmuramic
acid (Neu5Ac) is the most common (1). These negatively
charged sugars are important in mammalian cell – cell recogni-
tion when incorporated as the terminal component of surface
glycoconjugates (2). Tumor-derived sialic acids can disable the
killing mechanisms of effector immune cells (3). Expression of
sialic acids on the bacterial cell surface provides a way to escape
the host immune system (4). A variety of virus – host interac-
tions also involve sialic acids (5). The biosynthesis of sialic acids
starts with hydrolytic epimerization of N-acetylglucosamine
(GlcNAc), catalyzed by UDP-GlcNAc 2-epimerase and produc-
ing N-acetylmannosamine (ManNAc), which then reacts with
phosphoenolpyruvate to form Neu5Ac (6, 7). Glucose and
mannose are epimers at C2, and hence the enzyme is a 2-epi-
merase. In mammals, ManNAc is phosphorylated at C6 by the
kinase part of the bifunctional enzyme GNE, and an additional
step is required to separate the 9-phosphate group from Neu5Ac.
Subsequently, the sugars are activated by CMP-sialic acid syn-
thetases, ready to be presented on the cell surface. The down-
stream product CMP-Neu5Ac also acts as a feedback inhibitor
to regulate the GNE activity. Impaired inhibition can result in
sialuria (8).

The Gram-negative cocccobacillus Acinetobacter baumannii
is an opportunistic pathogen that causes nosocomial (i.e. origi-
nating in the hospital) urinary tract and bloodstream infec-
tions, ventilator-associated pneumonia, and meningitis (9).
The emergence of A. baumannii strains that can resist treat-
ment with antibiotics, as a result of acquisition and expression of
β-lactamase genes, has posed a significant problem in elimi-
nating the bacteria (10). Secretion of capsular polysaccharide
and formation of biofilm can further protect the bacteria from
antibiotics and the host immune system (11). Besides, the
genome of some A. baumannii strains, as well as those of other
pathogenic strains of Pseudomonas aeruginosa, Escherichia
coli, and Neisseria meningitidis, for example, contains genes
that encode enzymes for sialic acid biosynthesis (12). The bac-
terial enzymes in the sialic acid pathway are named NeuC,
NeuB, and NeuA, which correspond to UDP-GlcNAc 2-epi-
merase, sialic acid synthetase, and CMP-sialic acid synthetase
(Fig. 1). The crystal structures of prokaryotic NeuB and NeuA
have been solved for N. meningitidis (PDB codes 1XUU and
1EYR) (13, 14). The structure of a mammalian CMP-Neu5Ac
synthetase is also known (PDB code 1QWJ) (15). However, no
NeuC structure has been solved until now. Only the related
structure of human GNE is available (PDB code 4ZHT) (16).

Whereas the substrate for nonhydrolyzing epimerase and
hydrolyzing epimerase is the same, in this case UDP-GlcNAc,
the product of the former is UDP-ManNAc and that of the
latter is α-ManNAc plus UDP. As a hydrolyzing enzyme, NeuC
is different from the nonhydrolyzing epimerase for bacterial
cell wall manufacturing (6). Despite the limited homology, the
Tetrameric hydrolyzing UDP-GlcNAc 2-epimerase

![Figure 1. Sialic acid biosynthesis in bacteria.](image)

**Figure 1.** Sialic acid biosynthesis in bacteria. A schematic drawing of Neu5Ac is shown with each carbon atom numbered. The green part on left comes from Neu5Ac, and the cyan part on right is from PEP. The pathway starts by converting UDP-GlcNAc into UDP and ManNAc by the epimerase NeuC (or SiaA). It is followed by reacting the ManNAc with PEP and activation of the resulting Neu5Ac with CTP, catalyzed by sialic acid synthetase (NeuB) and CMP-sialic acid synthetase (NeuA).

Two classes of epimerases share a common protein fold and dimeric organization (16). Crystal structures of the nonhydrolyzing enzyme are known for at least 10 species (PDB codes 1F6D, 1O6C, 1V4V, 3BEO, 3DZC, 3OT5, 4HWG, 4NEQ, 5DLD and 5ENZ). Each protomer contains two Rossmann-like domains. The binding of UDP-GlcNAc to an allosteric site stabilizes the closed, active conformation of the enzyme, whereas the open form is not active (17). Open and closed conformations are believed to affect the hydrolyzing enzyme activity as well, but so far the precise relationship remains unclear. In addition to dimer, the epimerase part of human GNE formed a tetramer in the crystal, which contained UDP and CMP-Neu5Ac (16). The feedback inhibitor binds to the dimer–dimer interface and locks the tetramer into a tightly closed and inactive conformation. This is distinct from the activation mechanism of the nonhydrolyzing enzyme through a closed conformation.

To investigate the protein conformations of bacterial hydrolyzing UDP-GlcNAc 2-epimerase, as well as the underlying catalytic and regulatory mechanisms, we cloned, expressed, purified, and crystallized the NeuC protein from *A. baumannii*, and we solved its structure. Unexpectedly, not only did NeuC turn out to be a dimer, but the dimers also assemble into a tetramer, which bears striking similarity to that of human GNE. The substrate-binding mode was also investigated by soaking experiments. Analyses of the crystal structures suggest a conserved catalytic mechanism employed by NeuC and GNE.

**Results**

**Overall structure and tetramer formation**

The two NeuC protomers in the C222, crystal were modeled with 370 and 366 amino acid residues. The structure was refined to acceptable polypeptide geometry and low R-values (Table 1). Each protomer comprises two Rossmann-fold domains. The N-terminal domain contains seven parallel β-strands (β1–β7) connected by six α-helices (α1–α6) with a topology of +1x/+1x/−3x/−1x/−1x/−1x. The order of strand arrangement is β3-β2-β1-B4-β5-β6-β7. The C-terminal domain contains six parallel β-strands (β8–β13) linked by five α-helices (α9–α13) with a similar topology of +1x/+1x/−3x/−1x/−1x/−1x and it is connected to the N-terminal domain by a loop between helices α7 (as part of the N domain) and α8 (in the C domain). The C-terminal segment of the protein, which includes an additional helix α14, is associated with the N-terminal domain (Fig. 2A). The loop between helices α13 and α14 in both protomers lacked clear electron density in the Fourier maps, suggesting high flexibility in this region. Moreover, the two protomers showed different overall conformation: one is open and the other is closed (Fig. 2B). If they are superimposed directly, the root-mean-square deviation (r.m.s.d.) is 2.73 Å for 359 matched pairs of Ca atoms. However, if the N- and C-terminal domains are superimposed separately, the r.m.s.d. are significantly reduced to 0.193 and 0.466 Å for 182 and 140 pairs of Ca, respectively, indicating that the conformational change is largely the result of rigid-body domain movements.

The two NeuC protomers form a dimer in a way similar to those of bacterial nonhydrolyzing UDP-GlcNAc 2-epimerase and human GNE. The dimerization mainly involves N-terminal domain association in a highly symmetrical manner (Fig. 3A). By excluding the C-terminal domain, the NeuC dimer can turn 180° and superimpose on itself with a r.m.s.d. of 0.197 Å for 364 matched pairs of Ca atoms (Fig. S1). It is significantly lower than the 3.47-Å r.m.s.d. for 716 pairs of Ca if the entire dimer is compared, apparently because of the conformational difference between the open and closed protomers. Dimer formation buries 1970-Å² surface areas on each protomer, involving at least 40 amino acid residues. The interface is contributed mainly by helices α3, α4, and α5, as well as parts of helix α6 and the C-terminal segment (Fig. 3B). It is centered at a hydrophobic core with the side chains of Val-76, Met-79, Leu-83, Ala-87, Leu-108, Ala-113, Leu-115, Ile-116, Met-117, Tyr-134, Ile-138, Ala-141, and Met-145 (Fig. 3C). Buried deep in this core are the hydrogen-bonded side chains of Gln-112 and Gln-112* (asterisks denote residues from a different protomer). At the rim of the interface, four salt bridges are formed between two pairs of Lys-77–Asp-88* and Arg-159–Asp-377* (Fig. 3B). Besides these major interactions, the side chain of Gln-162 is hydrogen-bonded to the backbone of Tyr-376* (CO) and Leu-378* (NH), and the side chain of Phe-375 is in contact with those of Ile-129* and Leu-163*. Near the C terminus, the side chains of Leu-378–Leu-378* are only 3.9 Å apart.

The NeuC dimer further forms a tetramer with another dimer related by a crystallographic dyad symmetry. For clarity, a model of the tetramer is constructed by renaming the symmetry-related protomers A* and B* to protomers C and D. It is interesting to note that the organization of NeuC tetramer is also similar to that of human GNE, with 222 point-group symmetry. A tetrameric model of human GNE likewise obtained via crystallographic dyad symmetry shows an r.m.s.d. of 1.14 Å from NeuC for 182 matched pairs of Ca (Fig. 3B). Besides these major interactions, the side chain of Gln-162 is hydrogen-bonded to the backbone of Tyr-376* (CO) and Leu-378* (NH), and the side chain of Phe-375 is in contact with those of Ile-129* and Leu-163*. Near the C terminus, the side chains of Leu-378–Leu-378* are only 3.9 Å apart.

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Table 1
Data collection and refinement statistics of the NeuC crystals

|                         | Native-NeuC | NeuC-complex 1 | NeuC-complex 2 |
|-------------------------|-------------|----------------|----------------|
| **Data collection**     |             |                |                |
| Space group             | C 2 2 2     | P 2 2 2 2     | P 2 2 2 2     |
| Unit cell a, b, c (Å)   | 86.5, 148.7, 125.7 | 85.6, 126.1, 146.0 | 86.9, 147.7, 125.0 |
| Resolution (Å)          | 28.3–2.0 (2.07–2.0)* | 27.8–2.5 (2.59–2.50) | 30.0–2.39 (2.48–2.39) |
| Average I/e (I)         | 27.28 (3.82) | 14.58 (2.39)   | 9.22 (3.43)    |
| Completeness (%)        | 99.7 (98.0) | 97.7 (98.7)    | 97.7 (99.6)    |
| Redundancy              | 3.2 (5.1)   | 3.9 (3.51)     | 6.5 (6.6)      |
| $R_{	ext{merge}}$      | 0.049 (0.428) | 0.083 (0.478)  | 0.094 (0.65)   |
| **Refinement**          |             |                |                |
| No. of reflections      | 54,762      | 53,702         | 32,251         |
| $R_{	ext{work}}/R_{	ext{free}}$ | 0.166/0.212 | 0.204/0.257    | 0.171/0.211    |
| $B$-factors (Å$^2$)     | 48.04       | 50.75          | 67.69          |
| r.m.s.d.                |             |                |                |
| Bond lengths (Å)        | 0.009       | 0.003          | 0.004          |
| Bond angles (°)         | 1.01        | 0.69           | 0.77           |
| Ramachandran (%)        | 95          | 95             | 97             |
| Favored (%)             |             |                |                |
| Outliers (%)            | 0.41        | 0.21           | 0.14           |
| **PDB codes**           | S5LR        | S5LT           | 5XVS           |

$^*$Statistics for data from the highest-resolution shell are shown in parentheses.

$^a$ $R_{	ext{merge}} = \left(\sum|I_{\text{obs}}| - |I_{\text{calc}}|\right)/\left(\sum|I_{\text{calc}}|\right)$, where the average intensity (I) is taken over all symmetry-equivalent measurements, and $I_{\text{calc}}$ is the intensity for any given reflection.

$\text{work}$ set was collected and processed in space group $P2_12_12_1$, whereas $C222_1$ was used in the refinement.

$R_{	ext{work}} = \left(\sum|F_{\text{obs}}| - |F_{\text{calc}}|\right)/\left(\sum|F_{\text{calc}}|\right)$, where $F_{\text{obs}}$ and $F_{\text{calc}}$ are the observed and calculated structure factor amplitudes, respectively.

$^b$$R_{	ext{free}}$ value was calculated by using only an unrefined subset of the reflection data (5%).

Figure 2. Overall structure of NeuC. A, in the ribbon diagram, the model of a NeuC protomer is colored from the N to C terminus by a spectrum of blue to red. The cylinders and arrows represent $\alpha$-helices and $\beta$-strands as they are labeled. The loop connecting helices $\alpha$13 and $\alpha$14 is incomplete. Also shown is a surface representation of the other protomers in a tetramer. B, two protomers in the native crystal are superimposed. The open protomer is colored cyan/pink/magenta for helices/strands/loops. The closed protomer is colored red/yellow/green. The bound sulfate ions (with cyan/yellow sulfur atoms in the open/closed protomers) are shown as spheres.

and 24 in B/D) are involved. The dimer–dimer interface is mainly contributed by the $\beta2$–$\alpha2$ loop, strand $\beta3$, the $\beta3$–$\alpha3$ loop, and helix $\alpha3$. A central hydrophobic core is formed by the nonpolar side chains of Leu-67, Val-81, and Leu-84 from all four protomers (Fig. 3D). The other interactions are mostly polar, including a salt bridge between the Glu-61 and Arg-92* side chains and a backbone hydrogen bond between Leu-66 (CO) and Ser-68* (NH; Fig. 3E). Depending on the conformation being open or closed, the side chain of Glu-64 forms alternate hydrogen bonds with those of Ser-69*, Thr-71*, and Ser-73*. In addition, Pro-43 of protomer A (or C) is in contact with Thr-247* and Lys-251* of protomer D (or B).

Substrate soaking and ligand-binding mode

In addition to 572 water molecules, the native NeuC crystal structure contains two lithium ions and two sulfate ions in the solvent model (Fig. S3). These ions presumably come from the crystallization buffer. Each bound lithium ion is coordinated by four peptide carbonyl groups in the $\alpha1$–$\beta2$ loop, adjacent to the N terminus of each protomer (Fig. S3A). The sulfate ions are each bound to Ser-290 at the N terminus of helix $\alpha12$ and the positively charged side chain of Arg-309, both in the C-terminal domain (Fig. S3B). The sulfate-binding site in NeuC correlates very well with the location of the pyrophosphate group of UDP in the active site of human GNE.

To investigate the precise substrate-binding mode of NeuC, co-crystallization experiments with UDP-GlcNAc were carried out but failed to yield suitable crystals for X-ray data collection. Neither did longer-time soaking experiments with native NeuC crystals turn out Fourier maps with significant density for the substrate. Interestingly, the space group of the crystals was transformed from $C222_1$ to $P2_12_12_1$ after the soaking. The new crystals contained one tetramer in an asymmetric unit. When the soaking time was reduced to about 1 min, a data set was obtained from a crystal with space group $P2_12_12_1$, which showed densities for two bound UDPs. Finally, by limiting the soaking time to seconds, some density for the GlcNAc moiety was seen. The diffraction images could only be processed in $P2_12_12_1$, but the resulting data set contained mostly zero or negative intensities when the sum of $h + 1$ was odd. The electron density map based on a preliminarily refined model
showed two virtually identical dimers, and the crystal was considered isomorphous to the native. Consequently, those odd reflections were omitted, and the indices $k$ and $l$ were switched to make a C222$_1$ data set.

The NeuC tetramer of the P2$_1$2$_1$2$_1$ crystal was compared with that of the native C222$_1$ crystal. The r.m.s.d. between 1103 matched pairs of $C\alpha$ atoms is 0.240 Å. Again, the C-terminal domains of protomers A and C were excluded upon structural superposition for their outstanding deviations. These protomers also adopted the open conformation in the P2$_1$2$_1$2$_1$ crystal, but they became even more open than the protomer A in the C222$_1$ crystal (Fig. S2B). The bound sulfate ions remained with the open protomers A and C, whereas a bound UDP molecule was observed in each active site of the closed protomers B and D (Fig. 4, A and B). The electron densities for the sugar and base of the UDP are comparatively weaker than those for the pyrophosphate group. In protomer B, the side chain of Arg-11 is in close contact with the uracil, causing disorder in this region. However, it is rotated and directed away from the active site in protomer D (Fig. S4A). In the substrate-soaked C222$_1$ crystal (C$\alpha$ r.m.s.d. = 0.135 Å from the native) UDP was found in the active site of each protomer (Fig. 4, C and D). The binding of UDP to the open protomer A appears to be weaker, in which the Arg-11 side chain is away from the uracil base (Fig. S4B). The closed protomer B contains an additional GlcNAc moiety.

As shown in Fig. 5A, the $\beta$-phosphate group of UDP interacts with the protein in a virtually identical way as that for the sulfate in the native structure, making four hydrogen bonds to Ser-290 and Arg-309. The $\alpha$-phosphate makes additional hydrogen bonds to the side chains of Arg-11 and His-210. The planar guanidinium group of Arg-11 appears to stack with the uracil base, which in turn stacks with the side chain of Tyr-275, making an arginine–uracil–tyrosine sandwich. The uracil group forms two hydrogen bonds to the backbone NH and CO groups making some variations of the hydrogen bonds to the uracil, which requires tautomerization, were also observed (Fig. S5). Most of the UDP-binding residues in human GNE except Ser-23 are found in NeuC (Fig. S6). The UDP interactions with NeuC are fewer than those observed in the GNE structure, probably because the active site of NeuC needs structural rearrangements to accommodate the substrate, but the conformational changes are limited by the crystal lattice packing. The enzyme can form similar hydrogen bonds to the $\beta$-phosphate and the ribose OH groups using the Arg-104 and Glu-295 side chains if they are properly reoriented. In Fig. 5B, the GlcNAc part of the substrate is disconnected from UDP. The N-acetyl
group makes a hydrogen bond to the β-phosphate of UDP and also interacts with the side chains of Leu-16 and Leu-101. The OH groups of the sugar are hydrogen-bonded to the side chains of Glu-128, Arg-139, and Ser-290, as well as the backbone CO of His-125. The side chain of Asp-103 lies adjacent to the C1 and O5 of GlcNAc, but Asp-135 does not interact directly with the sugar. Most GlcNAc-binding residues of NeuC are found in GNE as well (Fig. S7).

**Effects of site-specific mutation on catalytic activity**

The catalytic mechanism of hydrolyzing UDP-GlcNAc 2-epimerase has been extensively studied for human GNE (18), and the biochemical characterization has also been reported for two bacterial enzymes from *E. coli* (NeuC) and *N. meningitidis* (SiaA) (19, 20). Strictly speaking, these hydrolyzing enzymes are not true epimerases as are the nonhydrolyzing enzymes that catalyze reversible epimerization of UDP-GlcNAc and UDP-ManNAc. The hydrolyzing epimerization is believed to proceed by a two-base mechanism. The substrate first undergoes anti elimination in which the first base deprotonates the C2 carbon of the sugar, forming the 2-acetamidoglucal intermediate and UDP. It is followed by syn hydration, catalyzed by the second base, on the other side of the sugar ring (18). The GlcNAc-binding mode in *A. baumannii* NeuC suggests that the β-phosphate of UDP may act as the first base, which is probably assisted by the invariant Ser-290. Conserved in all hydrolyzing UDP-GlcNAc 2-epimerases but not found in the nonhydrolyzing enzymes, Arg-104 may bind to the β-phosphate and serve to stabilize the leaving UDP (16). The reduction and lack of catalytic activity in the human GNE mutants S302A and R113A underscore the functional importance of the equivalent Ser-290 and Arg-104 in NeuC. From the decrease of $k_{cat}$ value by 150-fold in the GNE mutant D112A, it is likely that Asp-103 of NeuC plays an important role in catalysis, probably as the second base.

However, the catalytic role of Asp-135 in NeuC is not clear, although the corresponding GNE mutant D143A completely lost activity. The NeuC-GlcNAc binding mode cannot explain another inactive GNE mutant E134A, as the equivalent residue is His-125 in NeuC. In a previous model, the Glu-134 side chain of GNE made a hydrogen bond to the 4-OH group of ManNAc...
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Discussion

Because most active-site residues are conserved in NeuC and GNE, these two enzymes as well as other homologues are supposed to share a common catalytic mechanism. The observation of a bound GlcNAc in the active site of NeuC allows a revised mechanism to be proposed (Fig. 6). First, because the β-phosphate of UDP is located above the sugar plane and closer to C2 than C1, upon Arg-104–assisted bond breaking of UDP from GlcNAc, the phosphate may serve as the first base to abstract the C2 proton from the sugar, probably assisted by the nearby Ser-290. Second, Asp-103 may have another role than serving as the second base. Because the side-chain oxygen atoms of Asp-103 are in contact with the O5 of GlcNAc at distances of 2.9 and 3.1 Å, the negatively charged side chain can stabilize the positive charge of an oxocarbenium intermediate that may be transiently present after UDP elimination. Third, although the His-125 side chain of NeuC does not interact directly with the sugar, when it is rotated to a similar conformation as Glu-134 in GNE (Fig. S7), the imidazole (or carboxyl) group will be positioned below the sugar ring, where few polar residues are found, and serve as a suitable base for C2 re-protonation on the other side. Finally, according to the observed GlcNAc-binding mode, the base that activates a water molecule to attack C1 in the last step can be Asp-103 or His-125, rather than UDP and Ser-290. The involvement of oxocarbenium intermediate is the most likely mechanism of retaining glycosyltransferases (21). The hydrolyzing epimerases are similar to the retaining enzymes in some aspects, as the hydration step can be regarded as transfer of the sugar residue to a water molecule.

The similarity between NeuC and GNE is not limited to the active-site configuration but extends further to the conserved way of dimer and tetramer formations. Even the dispositions of the C-terminal segments are similar (Fig. S8). However, in a NeuC dimer, the loop beyond helix α14 turns inward and interacts with other residues near the proteomer–protomer interface. The average B-value of the last 12 amino acid residues is 43.7 Å², lower than the overall B-value of 47.8 Å² for the protein. In comparison, the corresponding loop in GNE has a much higher B-value than the overall average, indicating significant flexibility (16). It lacks specific interactions with the epimerase part and is supposed to swing out in order to connect with the kinase part, which also forms a dimer. Regarding the tetramer, the three nonpolar amino acids Leu-67, Val-81, and Leu-84 in the β3–α3 loop and helix α3 of NeuC, which make up the hydrophobic core of the dimer–dimer interface, are equivalent to Val-76, Leu-90, and Val-93 in GNE (Fig. 7). Although not identical, these side chains pack snugly against one another in a similar way at the interface, which is strengthened by a number of polar interactions. A previous study showed that rat GNE

(16). However, in the complex structure of NeuC, the side chain of His-125 is not in direct contact with GlcNAc, but the backbone CO makes a hydrogen bond to the sugar’s 6-OH group. The 4-OH is hydrogen-bonded to the Glu-128 side chain instead. To investigate the roles of these two residues in substrate binding and catalysis, two NeuC mutants H125A and E128A were produced and their catalytic parameters measured, along with the WT enzyme (Table 2). Compared with human GNE, the bacterial enzyme showed a lower $k_{cat}$ by about 4-fold and a much higher $K_m$ by about 30-fold, under the experimental conditions. The lower activity of NeuC than GNE might imply that sialic acid biosynthesis is not an essential pathway for bacterial growth, but the nine-carbon sugar is an important metabolite in mammals, where the production is also stringently controlled by feedback inhibition. Compared with WT NeuC, the mutant H125A showed a reduced $k_{cat}$ value to nearly one-half and a 2.7-fold higher $K_m$. The overall reduction of activity to about 20% suggests that His-125 plays an essential role in both substrate binding and catalysis, and it does so not only by making a backbone hydrogen bond to the sugar, but is not required for catalysis.

Table 2

| Catalytic parameters of A. baumannii NeuC and human GNE | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|------------------------------------------------------|----------|--------|---------------|
| NeuC wildtype                                        | 2.86     | 0.98   | 2.9           |
| H125A                                                | 1.60     | 2.61   | 0.61          |
| E128A                                                | 41.2     | 3.91   | 10.5          |
| GNE wildtype (a)                                     | 11.8     | 0.0331 | 356           |
| D112A                                                | 0.076    | 0.0005 | 152           |
| E113A                                                | ND⁡     | ND⁡    | ND⁡           |
| E134A                                                | ND⁡     | ND⁡    | ND⁡           |
| D143A                                                | ND⁡     | ND⁡    | ND⁡           |
| S302A                                                | 0.791    | 0.0121 | 65            |

(a) These data are taken from Ref. 16.
(b) ND means not determined.

Figure 6. Proposed catalytic mechanism of NeuC. UDP is dissociated from GlcNAc and, attracted by Arg-104, positioned above the sugar ring upon binding to the enzyme. Asp-103 is believed to stabilize the transient oxocarbenium intermediate. UDP then acts as the first base, possibly mediated by Ser-290, to deprotonate the C2 of GlcNAc, forming the 2-acetamidoglucal intermediate. The reaction concludes with re-protonation of C2 on the other side and attack at C1 by an activated water molecule, in which a second and/or a third base (probably His-125 and/or Asp-103) should be involved.
protomers can reversibly associate into dimers and tetramers, whereas the presence of UDP-GlcNAc promoted tetramer formation (22). Crystallization of the epimerase part of human GNE as a tetramer was required (20). Neu5Ac, which participated in the dimer–dimer interactions (16). In the absence of ligands, it existed predominantly as a dimer in solution. Interestingly, in this study, Neu5Ac was purified as a tetramer in the absence of UDP-GlcNAc, as shown by the gel-filtration profile (Fig. 7). Because the three nonpolar residues are found in the Neu5Ac proteins from other species, including E. coli, presumably they function as similar tetramers as well. However, a sequence comparison of N. meningitidis SiaA with GNE and Neu5Ac shows that the tetramer-forming interface may be absent in SiaA. In addition to a gap near the N terminus of helix α3 in the aligned sequence (Fig. 7), the two nonpolar residues in the middle of this helix are replaced by asparagine and threonine. Although an equivalent isoleucine residue to Leu-67 of Neu5Ac (Val-76 of GNE) is found in SiaA, it may be insufficient to constitute a hydrophobic core between the two dimers. Consequently, SiaA is more likely to function as a similar tetramer as well.

Despite the similarity in oligomerization and catalytic mechanism, there are a few significant structural and functional differences between Neu5Ac and GNE. The Neu5Ac protomers showed both open and closed conformations, whereas the GNE protomers were all closed (16). Because the N-terminal domain is arranged much the same way in both Neu5Ac and GNE tetramers, apparently, formation of tetramer does not restrict the movements of the C-terminal domain for substrate binding and product release. A previous study showed that the E. coli enzyme was not inhibited by CMP-Neu5Ac (19). In human GNE, the side chains of three positively charged residues Arg-263, Arg-266, and Lys-267 from helix α10 at the dimer–dimer interface bind to the negatively charged CMP-Neu5Ac. The equivalents are Lys-251, Leu-254, and Asp-255 in Neu5Ac, among which only one is positively charged. In GNE, the guanidine group of Arg-263 is salt-bridged to both the 1-carboxylate and the phosphate groups of the inhibitor. The single amino group of Lys-251 in Neu5Ac would make the bonds much weaker, which alone is probably unable to lock the tetramer in a tightly closed conformation. Inspection of the pocket for sugar mononucleotide binding at the dimer–dimer interface of Neu5Ac suggests a less polar environment for the ribose phosphate moiety, which fits well with a hydantoin ring, and a larger space for the cytosine base, likely sufficient for a purine group. Thus the cleft at the dimer–dimer interface may accommodate two cross-linked hydantoin molecules, each with an indole group attached. Hopefully, Neu5Ac can be locked into an inactive closed tetramer like that of GNE but by less polar interactions than salt bridges.

**Experimental procedures**

**Cloning, expression, and purification of Neu5Ac**

The neu5Ac gene fragment of A. baumannii was amplified and cloned into pET21b (+) vector, for the optimized expression of recombinant protein with an N-terminal His tag. Target protein was thereafter produced in E. coli BL21 (DE3) cell culture with 0.5 mM isopropyl 1-thio-α-D-galactopyranoside induction for 16 h at 293 K. Cell pellet was harvested by centrifugation and then resuspended in a binding buffer of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazole.

After disruption of the cell suspension by sonication, the clarified supernatant was purified by Ni2+–affinity and size-exclusion chromatography. The purified Neu5Ac protein was stored in a buffer of 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol, and 2 mM tris(2-carboxyethyl)phosphine, and concentrated to 5 mg ml−1 as determined by the method of Bradford for crystallization experiments.

**Crystallization, soaking, and data collection**

Crystals of recombinant Neu5Ac protein were grown from hanging drops containing equal volumes of protein and reservoir solution, using the vapor diffusion method. The optimized reservoir consisted of 0.2 M lithium sulfate, 0.1 M Tris (pH 8.5), and 25% PEG 3350. Native crystals appeared within 3–4 weeks.
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at 277 K. The NeuC-UDP complex crystals were obtained by soaking the native crystals with 0.2 mM UDP-GlcNAc for 10–20 min. The crystals were then picked up and immersed briefly in the reservoir solution supplemented with 20% glycerol before flash-cooling in liquid nitrogen. X-ray diffraction data collection was performed at beamlines BL13B1 and BL13C1 in NSRRC (Hsinchu, Taiwan). The diffraction data were properly processed using the HKL2000 program suite (23) with data statistics as summarized in Table 1.

Structural determination and refinement

The native NeuC structure of A. baumannii was solved by molecular replacement with the PHENIX AutoMR (24) using the previously published human homologue structure (PDB code 4ZHT (16)) as a search model. The substrate-soaked NeuC complex structure was determined by CNS (25, 26), using a tetramer from the native NeuC structure as the search model. The UDP molecule was built into the extra electron density by using Coot (27). The crystal structures then underwent several rounds of manual model rebuilding and computational refinement with Coot and PHENIX. The figures were generated by PyMOL (28). Some refinement statistics and the PDB accession numbers are listed in Table 1.

Site-specific mutagenesis and activity measurement

The neuC gene from A. baumannii (ABNeuC) was constructed into the pET28a expression vector and then used as a template for site-directed mutagenesis to generate its derivatives through PCR with primer pairs specific for substituting the residues His-125 and Glu-128 with Ala. The pET28a-ABNeuC construct and its mutant derivatives were each transformed into E. coli BL21 for heterologous expression. Activity of UDP-GlcNAc 2-epimerase was detected via the procedures as described before (16, 29). Briefly, the reaction mixtures contained 50 mM Tris buffer (pH 7.5), 10 mM MgCl₂, 0.2 mM NADH, 2 mM phosphoenolpyruvate (PEP), 2 units of pyruvate kinase, 2 units of lactic dehydrogenase, and various concentrations of UDP-GlcNAc. The final volume of each reaction was 100 μl. The reaction was initiated by adding either 100 μg of ABNeuC, 100 μg of ABNeuC-H125A, or 40 μg of ABNeuC-E128A. The initial reaction velocities were measured at 37 °C for absorbance changes at 340 nm per min. Each data point was fitted into a Lineweaver-Burk plot to generate the kinetic parameters. The specific activity in this study was defined as the micro-molarity of product obtained per s/mg of enzyme.

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References

1. Chen, X., and Varki, A. (2010) Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5, 163–176 CrossRef Medline
2. Schauer, R. (2009) Sialic acids as regulators of molecular and cellular interactions. Curr. Opin. Struct. Biol. 19, 507–514 CrossRef Medline
3. Büll, C., den Brok, M. H., and Adema, G. J. (2014) Sweet escape: sialic acids in tumor immune evasion. Biochim. Biophys. Acta 1846, 238–246 Medline
4. Severi, E., Hood, D. W., and Thomas, G. H. (2007) Sialic acid utilization by bacterial pathogens. Microbiology 153, 2817–2822 CrossRef Medline
5. Neu, U., Bauer, J., and Stehle, T. (2011) Viruses and sialic acids: rules of engagement. Curr. Opin. Struct. Biol. 21, 610–618 CrossRef Medline
6. Tanner, M. E. (2005) The enzymes of sialic acid biosynthesis. Bioorg. Chem. 33, 216–228 CrossRef Medline
7. Hwang, T. S., Hung, C. H., Lee, C. F., Chen, G. T., Chang, L. S., Chen, S. F., Chen, Y. J., and Lin, C. H. (2002) Structural characterization of Escherichia coli sialic acid synthase. Biochem. Biophys. Res. Commun. 295, 167–173 CrossRef Medline
8. Hinderlich, S., Weidemann, W., Yardeni, T., Horstkorte, R., and Huizing, M. (2015) UDP-GlcNAc 2-epimerase/MannAc kinase (GNAE): a master regulator of sialic acid synthesis. Top. Curr. Chem. 366, 97–137 CrossRef Medline
9. Antunes, L. C., Visca, P., and Towner, K. J. (2014) Acinetobacter baumannii: evolution of a global pathogen. Pathog. Dis. 71, 292–301 CrossRef Medline
10. Maragakis, L. L., and Perl, T. M. (2008) Acinetobacter baumannii: epidermidis, antimicrobial resistance, and treatment options. Clin. Infect. Dis. 46, 1254–1263 CrossRef Medline
11. Limoli, D. H., Jones, C. J., and Wozniak, D. J. (2015) Bacterial extracellular polysaccharides in biofilm formation and function. Microbiol. Spectr. 2015, 3, MB-0011–2014 CrossRef Medline
12. Li, Y., and Chen, X. (2012) Sialic acid metabolism and sialyltransferases: natural functions and applications. Appl. Microbiol. Biotechnol. 94, 887–905 CrossRef Medline
13. Gunawan, J., Simard, D., Gilbert, M., Lovering, A. L., Wakarchuk, W. W., Tanner, M. E., and Strynadka, N. C. (2005) Structural and mechanistic analysis of sialic acid synthase NeuB from Neisseria meningitidis in complex with Mn²⁺, phosphoenolpyruvate, and N-acetamidomannosaminol. J. Biol. Chem. 280, 3555–3563 CrossRef Medline
14. Moinimm, S. C., Gilbert, M., Dombrowski, D., To, R., Wakarchuk, W. W., and Strynadka, N. C. (2001) Structure of a sialic acid-activating synthetase, CMP-acetylneuraminic synthetase in the presence and absence of CDP. J. Biol. Chem. 276, 8190–8196 CrossRef Medline
15. Krapp, S., Münster-Kühnel, A. K., Kaiser, J. T., Huber, R., Tiralongo, I., Gerardy-Schahn, R., and Jacob, U. (2003) The crystal structure of murine CMP-5'-N-acetylneuraminic acid synthetase. J. Mol. Biol. 334, 625–637 CrossRef Medline
16. Chen, S. C., Huang, C. H., Lai, S. I., Yang, C. S., Hsiao, T. H., Lin, C. H., Fu, P. K., Ko, T. P., and Chen, Y. (2016) Mechanism and inhibition of human UDP-GlcNAc 2-epimerase, the key enzyme in sialic acid biosynthesis. Sci. Rep. 6, 23274 CrossRef Medline
17. Chen, S. C. H., Yang, C. S., Liu, S. J., Kuan, S. M., and Chen, Y. (2014) Crystal structures of the archaeal UDP-GlcNAc 2-epimerase from Methanocaldococcus jannaschii reveal a conformational change induced by UDP-GlcNAc. Proteins 82, 1519–1526 CrossRef Medline
18. Chou, W. K., Hinderlich, S., Reutter, W., and Tanner, M. E. (2003) Sialic acid biosynthesis: stereochemistry and mechanism of the reaction catalyzed by the mammalian UDP-N-acetylglucosamine 2-epimerase. J. Am. Chem. Soc. 125, 2455–2461 CrossRef Medline
19. Vann, W. F., Daines, D. A., Murkin, A. S., Tanner, M. E., Chaffin, D. O., Rubens, C. E., Vionnet, J., and Silver, R. P. (2004) The NeuC protein of Escherichia coli K1 is a UDP N-acetylglucosamine 2-epimerase. J. Bacteriol. 186, 706–712 CrossRef Medline
20. Murkin, A. S., Chou, W. K., Wakarchuk, W. W., and Tanner, M. E. (2004) Identification and mechanism of a bacterial hydrolyzing UDP-N-acetylglucosamine 2-epimerase. Biochemistry 43, 14290–14298 CrossRef Medline
21. Lairos, L. L., Henriksson, B., Davies, J. G., and Withers, S. G. (2008) Glycosyltransferases: structures, functions, and mechanisms. Annu. Rev. Biochem. 77, 521–555 CrossRef Medline
22. Ghaderi, D., Strauss, H. M., Reinke, S., Cirak, S., Reutter, W., Lucka, L., and Hinderlich, S. (2007) Evidence for dynamic interplay of different
oligomeric states of UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase by biophysical methods. J. Mol. Biol. 369, 746–758 CrossRef Medline

23. Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 CrossRef Medline

24. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 CrossRef Medline

25. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. 54, 905–921 CrossRef Medline

26. Brünger, A. T. (2007) Version 1.2 of the Crystallography and NMR system. Nat. Protoc. 2, 2728–2733 CrossRef Medline

27. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132 CrossRef Medline

28. DeLano, W. L. (2016) The PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC, New York

29. Hinderlich, S., Stäsche, R., Zeitler, R., and Reutter, W. (1997) A bifunctional enzyme catalyzes the first two steps in N-acetyleneuraminic acid biosynthesis of rat liver. J. Biol. Chem. 272, 24313–24318 CrossRef Medline

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