Glycobiology and Extracellular Matrices:

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The Polysialic Acid-specific *O*-Acetyltransferase OatC from *Neisseria meningitidis* Serogroup C Evolved Apart from Other Bacterial Sialate *O*-Acetyltransferases*[S]◆

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Neisseria meningitidis **serogroup C is a major cause of bacterial meningitis and septicaemia. This human pathogen is protected by a capsule composed of α2,9-linked polysialic acid that represents an important virulence factor. In the majority of strains, the capsular polysaccharide is modified by *O*-acetylation at C-7 or C-8 of the sialic acid residues. The gene encoding the capsule modifying *O*-acetyltransferase is part of the capsule gene complex and shares no sequence similarities with other proteins. Here, we describe the purification and biochemical characterization of recombinant OatC. The enzyme was found as a homodimer, with the first 34 amino acids forming an efficient oligomerization domain that worked even in a different protein context. Using acetyl-CoA as donor substrate, OatC transferred acetyl groups exclusively onto polysialic acid joined by α2,9-linkages and did not act on free or CMP-activated sialic acid. Motif scanning revealed a nucleophile elbow motif (G*X*S[286]*X*GG), which is a hallmark of α/β-hydrolase fold enzymes. In a comprehensive site-directed mutagenesis study, we identified a catalytic triad composed of Ser-286, Asp-376, and His-399. Consistent with a double-displacement mechanism common to α/β-hydrolase fold enzymes, a covalent acetyl-enzyme intermediate was found. Together with secondary structure prediction highlighting an α/β-hydrolase fold topology, our data provide strong evidence that OatC belongs to the α/β-hydrolase fold family. This clearly distinguishes OatC from all other bacterial sialate *O*-acetyltransferases known so far because these are members of the hexapeptide repeat family, a class of acyltransferases that adopt a left-handed β-helix fold and assemble into catalytic trimers.**

Neisseria meningitidis (meningococcus) is a worldwide and devastating cause of epidemic meningitis and sepsis (1, 2). Despite progress in diagnostics, vaccination, and therapy, invasive meningococcal infections remain a prevalent disease with a mortality of 10% and high morbidity among survivors (3). Meningococci are usually found as commensal bacteria of the human upper respiratory tract, and only a minority of isolates cause systemic disease (4). Common to these invasive strains is the presence of a polysaccharide capsule that serves as an important virulence factor (5–7). Based on the capsular polysaccharide (CPS)[2] structure, meningococci were classified into 13 serogroups. However, only five serogroups (A, B, C, W-135, and Y) account for nearly all reported invasive diseases worldwide (1, 8). With the exception of serogroup A, these clinically important serogroups are decorated with polysialic acid (polySia) capsules (1, 9). Sialic acid refers to a family of nine-carbon sugars that are derived from *N*-acetylneuraminic acid (Neu5Ac). They are widely found as terminal sugars of glycoproteins and glycolipids on vertebrate cells but only in a limited number of pathogenic microorganisms, where they might have evolved to mimic host cell surfaces and to avoid immune attack (10, 11). The CPS of *N. meningitidis* serogroup B (NmB-CPS) is composed of α2,8-linked Neu5Ac, a homopolymer that is structurally identical to polySia found as a post-translational modification of the neural cell adhesion molecule NCAM (12, 13). In the case of serogroup C CPS (NmC-CPS), the sialic acid residues are joined by α2,9-linkages, whereas the CPS of serogroups W-135 (NmW-CPS) and Y (NmY-CPS) are Neu5Ac-containing heteropolymers composed of the disaccharide repeating units (→6)-α-D-Gal*p*-(1→4)-α-Neu5Ac-(2→) and (→6)-α-D-Glc*p*-(1→4)-α-Neu5Ac-(2→), respectively (14, 15). Interestingly, in serogroups C, W-135, and Y, but not in serogroup B, the Neu5Ac residues can be further modified by *O*-acetylation. In NmC-CPS, the acetyl groups are distributed exclusively between the hydroxyl groups located at C-7 and C-8 of Neu5Ac, whereas in NmW-CPS and NmY-CPS, *O*-acetylation is found at C-7 and C-9 (15–17). Studies from the United Kingdom showed capsule *O*-acetylation for 88 and 79% of the serogroup C and Y strains, respectively, whereas only 8% of the W-135 strains displayed *O*-acetylated capsules (18, 19). However, the biological impact of this modification with regard to pathogenicity is still unclear. For NmC-CPS and NmY-CPS, an inverse correlation between immunogenicity and the level of *O*-acetylation was observed, whereas no impact on immunoge-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: CPS, capsular polysaccharide(s); polySia, polysialic acid; Neu5Ac, *N*-acetylneuraminic acid; NmC, *N.meningitidis* serogroup C; NmW, *N.meningitidis* serogroup W-135; NmY, *N.meningitidis* serogroup Y; mAb, monoclonal antibody; MBP, maltose-binding protein; IMAC, immobilized metal affinity chromatography; OAc, *O*-acetylation.

nicity was found for *O*-acetylation of NmW-CPS (20, 21). These findings suggest that for serogroup C and Y meningococci, capsule *O*-acetylation may play a general role in masking immunogenic epitopes and thereby enabling escape from immune surveillance. The sialate *O*-acetyltransferases that catalyze the capsule *O*-acetylation might therefore constitute a virulence or fitness factor.

O-Acetyltransferases are very common in bacteria and modify a diverse set of substrates, including carbohydrates, amino acids, and xenobiotics. They are found as cytosolic, inner membrane-associated or integral membrane proteins and include members of different protein families. In 2004, we reported the cloning of the genes encoding the capsule-specific *O*-acetyltransferases of serogroup C and serogroups W-135 and Y (*oatC* and *oatWY*, respectively), which was at that time the first identification of genes that encode sialic acid-specific *O*-acetyltransferases (22). In serogroups W-135 and Y, an identical *O*-acetyltransferase gene was found, which might reflect the structural similarity between NmW-CPS and NmY-CPS, two heteropolymers that differ only in the orientation of the C-4 hydroxyl group of the hexose moiety. Both *oatC* and *oatWY* were located downstream of the polySia synthesis genes *siaA–D* within the capsule gene complex of serogroup C and W-135/Y meningococci, respectively. Whereas no homology to any known protein in the data bases was found for OatC, analysis of the primary sequence of OatWY revealed the presence of imperfect tandem repeats of the consensus sequence (LIV)(GAED)X_2(STAV)X, the hallmark of enzymes belonging to the hexapeptide repeat family of acyltransferases. Members of this family adopt a unique left-handed β-helix fold, and three identical subunits assemble into a catalytic trimer (23, 24).

Since 2004, several genes encoding sialate *O*-acetyltransferases have been identified in bacteria that contain sialic acid as a component of their CPS or lipo-oligosaccharide, such as *Escherichia coli* K1, *Campylobacter jejuni*, and type III group B streptococci (25–28). Notably, all these enzymes belong to the hexapeptide repeat family, suggesting that the specificity for sialic acid evolved exclusively within this protein family.

To investigate whether OatC is just an estranged member of the hexapeptide repeat family that lacks sequence homology or whether it is the first example of a sialate *O*-acetyltransferase belonging to a different protein family, we started a detailed biochemical characterization of this enzyme. Using serogroup C meningococcal spheroplast membranes as an enzyme source, Vann *et al.* (29) demonstrated that endogenous OatC transfers acetyl groups from acetyl-CoA to endogenous membrane-bound as well as to exogenous soluble NmC-CPS. However, reports on the isolation and further characterization of the enzyme are missing. In this study, we succeeded in purifying active recombinant OatC, which was found as a homodimer. A comprehensive site-directed mutagenesis study revealed the presence of a Ser-Asp-His catalytic triad with the serine located in a nucleophile elbow motif, a characteristic feature of α/β-hydrolase fold enzymes. The identification of a covalent acetyl-enzyme intermediate gave the first insight into the catalytic mechanism of OatC. Together, our data obtained by biochemical analysis of wild-type and mutant OatC provide strong evidence that OatC is a polySia-specific *O*-acetyltransferase that belongs to the α/β-hydrolase fold superfamily. This result demonstrates that OatC evolved apart from all other bacterial sialate *O*-acetyltransferases known so far.

EXPERIMENTAL PROCEDURES

Materials—Purified de-*O*-acetylated CPS from *N. meningitidis* serogroup C was kindly provided by Baxter. Purified CPS from *N. meningitidis* serogroups W-135 and Y were kindly donated by Pasteur Mérieux Connaught. CPS from *E. coli* K1 was purified as described (30) and kindly provided by T. Scheper (Institut für Technische Chemie, Universität Hannover, Hannover, Germany). Monoclonal antibody (mAb) P1.2 was kindly provided by J. Suker and I. Feavers (National Institute for Biological Standards and Control, Hertfordshire, United Kingdom). pET expression vectors and *E. coli* BL21(DE3) were obtained from Novagen. CMP-Neu5Ac, Neu5Ac, colominic acid, 5,5′-dithiobis(2-nitrobenzoic acid), acetyl-CoA, propionyl-CoA, and butyryl-CoA were purchased from Sigma.

Generation of OatC Expression Plasmids—All constructs for the expression of OatC variants were generated in the prokaryotic expression vector pET22b-Δ*Strep*, resulting in OatC variants with a C-terminal hexahistidine tag. pET22b-Δ*Strep* was generated by adapter ligation of oligonucleotides AKB42 and AKB43 into the BamHI/XbaI sites of pET22b-*Strep* (26), resulting in a vector that lacks the pelB leader sequence of pET22b and the Strep tag sequence of pET22b-*Strep.* Wild-type *oatC* was amplified by PCR using the primer pair MM269/MM271 and genomic DNA of *N. meningitidis* serogroup C as template. The PCR product was subcloned by the BamHI/XhoI sites into pET22b-Δ*Strep*. Constructs for the expression of the N-terminally truncated forms ΔN34-OatC and ΔN103-OatC were generated by PCR using the primer pairs AKB67/MM271 and AKB69/MM271, respectively. The PCR products obtained were subcloned into pET22b-Δ*Strep* as described above.

For expression of a hybrid protein composed of the first 34 amino acids of OatC fused to the maltose-binding protein (MBP) from *E. coli* (N34-MBP), a pET22b-based plasmid was generated in a two-step cloning approach. (i) The sequence encoding the first 34 amino acids of OatC was amplified by PCR using the primer pair MM269/AKB81 and wild-type *oatC* as template. The PCR product was subcloned into pET22b-Δ*Strep* by BamHI/XhoI sites, resulting in the plasmid $pOatC_{fusion}$. (ii) The MBP gene was amplified by PCR using the primer pair AKB72/AKB128 and the vector pMAL-c (New England Biolabs) as template. The resulting PCR product was subcloned into the HindIII/XhoI sites of $pOatC_{fusion}$.

For expression of MBP alone, the corresponding coding sequence was amplified by PCR using the primer pair AKB129/AKB128 and subcloned into the BamHI/XhoI sites of pET22b-Δ*Strep*. The identity of all generated constructs was confirmed by sequencing. The sequences of the indicated oligonucleotides are given in supplemental Fig. S1.

Site-directed Mutagenesis—Single amino acid substitutions were generated by PCR using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's guidelines. Wild-type *oatC* subcloned in pET22b-Δ*Strep* was used as template together with the primer pairs listed in sup-

plemental Fig. S1. BamHI/XhoI fragments of the PCR products obtained were subcloned in pET22b-Δ*Strep*, and the identity of the inserted PCR fragments was confirmed by sequencing.

Expression and Purification of Recombinant OatC—Freshly transformed *E. coli* BL21(DE3) cells were cultivated at 37 °C in 500 ml of Power Broth (AthenaES) containing 200 μg/ml carbenicillin. At A_{600} = 1.4, expression was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside. Thereafter, bacteria were grown at 15 °C for 20 h, harvested, and lysed by sonication. Recombinant proteins were isolated by immobilized metal affinity chromatography (IMAC) using 1 ml-HisTrap HP columns (GE Healthcare) equilibrated with binding buffer (50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 20 mM imidazole). After washing with 15 ml of binding buffer, proteins were eluted with a linear imidazole gradient (20–500 mM imidazole in binding buffer). Enzyme-containing fractions were pooled, concentrated by ultrafiltration, and loaded onto a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl.

Spectrophotometric Activity Assay—The enzymatic activity of purified OatC variants was determined in a spectrophotometric assay. The reaction was performed at 25 °C in a total volume of 100 μl containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20% (w/v) glycerol, 2 mM 5,5′-dithiobis(2-nitrobenzoic acid), 200 μg/ml purified NmC-CPS, and 1 mM acetyl-CoA. The reaction was initiated by adding 1.7 pmol of purified enzyme and monitored continuously at 405 nm in half-area 96-well plates (Greiner) using a PowerWave 340 microtiter plate spectrophotometer (BioTek).

Radioactive Incorporation Assay—For determination of OatC activity in crude bacterial lysates, a radioactive incorporation assay was performed. OatC variants were expressed in *E. coli* BL21(DE3) as described above. Bacteria from 1 ml of culture medium with A_{600} = 1 were resuspended in 25 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 100 μg/ml lysozyme), incubated for 15 min at 4 °C, and lysed by sonication. The enzyme reaction was performed at 25 °C in a total volume of 50 μl containing 20 μl of the soluble lysate fraction, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20 μg of NmC-CPS, and 9.25 kBq of [^{14}C]acetyl-CoA (GE Healthcare). The reaction was stopped after 15 min by spotting 10 μl of the reaction mixture on Whatman No. 3MM paper. Free radioactivity was removed by descending paper chromatography with running buffer (300 mM ammonium acetate buffer (pH 7.5) and 70% ethanol), and radioactivity incorporated into the chromatographically immobile NmC-CPS was quantified by scintillation counting. To determine the enzymatic activity of purified wild-type OatC, the reaction was performed in a total volume of 200 μl containing the same reaction mixture as described for the spectrophotometric assay supplemented with 37 kBq of [^{14}C]acetyl-CoA. At different time points, aliquots of the reaction mixture were analyzed as described above.

Detection of Acetyl-enzyme Intermediates—Purified enzyme (3.2 μg) was incubated in a final volume of 50 μl at 25 °C in the presence of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 9.25 kBq of [^{14}C]acetyl-CoA. After 5 min, one-half of the reaction mixture was removed. The remaining reaction mixture was supplemented with 20 μg NmC-CPS and incubated for 25 min. Enzyme reactions were stopped by adding an equal volume of loading buffer and separated by SDS-PAGE. Gels were analyzed by either phosphorimaging or combined Alcian blue/silver staining (31).

SDS-PAGE, Staining, and Immunoblotting—SDS-PAGE was performed under reducing conditions using 2.5% (v/v) β-mercaptoethanol. Silver staining, Coomassie staining, and Western blot analysis with anti-penta-His antibody (Qiagen) were performed as described previously (26).

Size-exclusion Chromatography—The quaternary structure of OatC variants was determined on a Superdex 200 HR 10/30 column. The column was equilibrated with 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl and calibrated with the following molecular mass standards (Sigma): thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Eluted proteins were monitored by absorbance at 280 nm.

Analysis of OatC Activity in Vivo—Isogenic NmC strains carrying different *oatC* variants were generated by homologous recombination. The downstream region of *oatC* that ought to provide the right anchor for homologous recombination was amplified using the primer pair FS1/FS2. The PCR product was subcloned into the ApaI sites of pBluescript II SK(+) (Stratagene), resulting in the plasmid pFS1. Subsequently, the SalI-restricted kanamycin resistance cassette of pUC4K (GE Healthcare) was inserted into the XhoI site of pFS1, resulting in pFS2. The kanamycin cassette was inserted to later select for transformants. Finally, wild-type and mutant *oatC* variants with an additional sequence stretch encoding a C-terminal hexahistidine tag were excised from the pET-based expression plasmids described above by sequential restriction. After restriction with HpaII and subsequent blunt ending with T4 DNA polymerase, the 1.5-kb fragment obtained was restricted with BamHI and inserted into the BamHI and EcoRV sites of pFS2. The resulting plasmids were transformed into *N. meningitidis* serogroup C wild-type strain 2120 (32) to allow homologous recombination. Correct insertion into the *oatC* locus was confirmed by PCR, Southern blotting, and sequence analysis. Meningococci were grown on chocolate agar, and 20 μl of a bacterial suspension (A_{600} = 0.15) was added to each well of a microtiter plate (Greiner) that had been coated with poly-D-lysine (Sigma). After drying, bacteria were fixed with 0.05% glutaraldehyde in phosphate-buffered saline. Capsule expression and modification by *O*-acetylation were analyzed in an enzyme-linked immunosorbent assay as described previously (32) using the following mAbs: mAb 924, which is specific for NmC-CPS (OAc^+ and OAc^- forms); mAb 1125, which is directed exclusively against the *O*-acetylated form of NmC-CPS (OAc^+); and mAb P1.2, which is directed against the surface protein PorA (33), as a loading control. *N. meningitidis* serogroup C strain 2948 lacking a functional *oatC* gene (22) was used as a negative control.

RESULTS

Purification of Active OatC—OatC was expressed in *E. coli* BL21(DE) with a C-terminal hexahistidine tag. The recombinant protein was isolated by IMAC and size-exclusion chromatography as described under "Experimental Procedures." Using

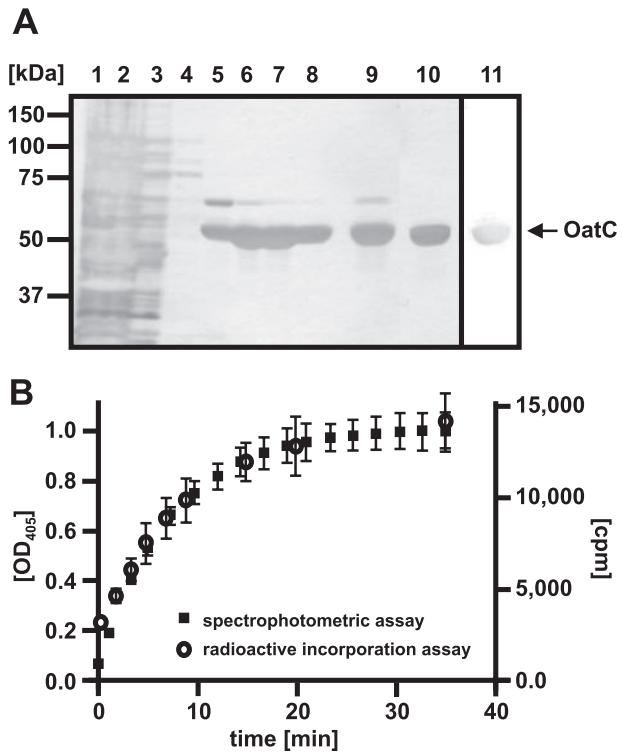

FIGURE 1. **Purification of OatC.** *A*, after expression in *E. coli* BL21(DE3), OatC carrying a C-terminal hexahistidine tag was isolated by IMAC on a Ni^{2+} chelating column followed by size-exclusion chromatography. Purification steps were monitored by 10% SDS-PAGE and silver staining, including aliquots of bacterial lysate (*lane 1*), flow-through of the IMAC column (*lane 2*), wash fractions (*lanes 3* and *4*), OatC-containing fractions eluted from the IMAC column (*lanes 5–8*), a pool of fractions 5–8 from the IMAC column (*lane 9*), and purified OatC after size-exclusion chromatography (*lane 10*). Purified OatC after size-exclusion chromatography was also analyzed on a Western blot stained with anti-penta-His antibody (*lane 11*). *B*, the enzymatic activity of purified OatC was determined in a spectrophotometric assay (data given as means ± S.D. of three independent experiments measured in triplicates) and a radioactive incorporation assay (data given as means ± S.D. of two independent experiments). A_{405} measured in the spectrophotometric assay is displayed on the *left y axis*, whereas the incorporated radioactivity is given as counts/min on the *right y axis*.

this two-step purification protocol, OatC was purified to homogeneity (Fig. 1*A*), yielding 24 mg of enzyme from 500 ml of bacterial culture. Silver staining revealed a single band consistent with a calculated molecular mass of 54.3 kDa, and the identity of this band was confirmed by Western blot analysis using anti-penta-His antibody (Fig. 1*A*).

To determine acetyltransferase activity, isolated OatC was incubated with [^{14}C]acetyl-CoA in the presence of purified NmC-CPS. After removal of free acetyl-CoA, incorporation of radiolabeled acetyl groups was measured by scintillation counting (Fig. 1*B*). In parallel, a spectrophotometric assay was established that monitored the transfer of acetyl groups from acetyl-CoA onto NmC-CPS by measuring the appearance of the free sulfhydryl group on released CoA with Ellman's reagent at 405 nm (Fig. 1*B*). In both assays, enzymatic activity was detected, and similar time courses were observed for the incorporation of acetyl groups and the release of CoA (Fig. 1*B*).

Donor and Acceptor Substrate Specificity of OatC—Using the spectrophotometric assay, we analyzed the acceptor substrate specificity of OatC. The enzymatic activity of purified OatC was monitored in the presence of monomeric Neu5Ac, activated

TABLE 1
Substrate specificity of OatC

Relative enzymatic activities of purified OatC were determined in the presence of different acceptor and donor substrates. The concentration of all acceptor substrates was normalized to equal sialic acid content (6.47 mM sialic acid residues) and thereby to an equal number of acceptor sites. The activity obtained for NmC-CPS and acetyl-CoA was set to 100%. Values are means ± S.D. from three independent experiments.

	Relative enzymatic activity
	%
Acceptor substrate	
NmC-CPS	100.0 ± 2.7
Neu5Ac	0.2 ± 0.6
CMP-Neu5Ac	0.3 ± 0.2
Colominic acid	0.4 ± 0.7
K1-CPS	0.3 ± 0.6
NmW-CPS	0.0 ± 0.4
NmY-CPS	0.3 ± 0.1
Donor substrate	
Acetyl-CoA	100.0 ± 2.4
Propionyl-CoA	21.4 ± 1.2
Butyryl-CoA	0.1 ± 0.1

Neu5Ac (CMP-Neu5Ac), the Neu5Ac-containing heteropolymers NmW-CPS and NmY-CPS, and polySia homopolymers composed of either α2,9-linked Neu5Ac residues (NmC-CPS) or α2,8-linked Neu5Ac residues (colominic acid and K1-CPS, the partially hydrolyzed and full-length CPS of *E. coli* K1, respectively). As summarized in Table 1, no enzymatic activity was detected for free Neu5Ac and CMP-Neu5Ac, indicating that OatC is specific for oligo- or polySia. However, of the tested polymeric substrates, only NmC-CPS served as an acceptor, demonstrating that OatC is highly specific for α2,9-linked polySia.

The donor substrate specificity of OatC was determined by measuring the enzymatic activity for NmC-CPS in the presence of acetyl-CoA and the alternative acyl donors propionyl-CoA and butyryl-CoA. Compared with acetyl-CoA, ~20% residual enzymatic activity was detected in the presence of propionyl-CoA, whereas no activity was observed with butyryl-CoA (Table 1).

OatC Assembles into Dimers Mediated by the First 34 Amino Acids—To study the oligomerization state of OatC, the purified enzyme was analyzed by size-exclusion chromatography on a Superdex 200 column. A single peak was obtained that corresponded to a molecular mass of 105 ± 1.6 kDa (Table 2), demonstrating that OatC is a homodimer with an apparent molecular mass that is in agreement with the calculated dimeric mass of 108.6 kDa.

Using the program HTH (34) and searching the Pfam Database of protein families (35), we identified a putative helix-turn-helix motif within the first 34 amino acids and a tetratricopeptide repeat spanning amino acids 71–104 of OatC. Because both motifs have been associated with protein-protein interactions (36–38), we asked whether the N-terminal part of OatC is involved in dimer formation. To address this question, the ΔN34-OatC and ΔN103-OatC variants, lacking the first 34 and 103 amino acids, respectively, were generated. After expression in *E. coli* BL21(DE3), both variants were purified to homogeneity by IMAC and size-exclusion chromatography (data not shown). Interestingly, both variants were found as monomers (Table 2), indicating that dimerization of OatC depends on the first 34 amino acids. Whereas no enzymatic activity was found

TABLE 2

Quaternary structures of OatC variants

The quaternary structures of purified OatC variants, the hybrid protein N34-MBP, and MBP were determined by size-exclusion chromatography. The molecular masses of the monomeric species were calculated from the respective amino acid sequences. The oligomeric state was calculated from the values obtained by gel filtration divided by the mass calculated for the monomer. Relative enzymatic activities of OatC variants were analyzed in the spectrophotometric assay, and the activity obtained for wild-type (WT) OatC was set to 100%. Values are means ± S.D. from three independent experiments. ND, not determined.

Protein variant	Molecular mass		Oligomeric state	Relative enzymatic activity
	Monomer (calculated)	Gel filtration		
	kDa			*%*
WT OatC	54.3	105.0 ± 1.6	2.0	100.0 ± 2.7
ΔN34-OatC	50.2	42.8 ± 0.8	0.9	24.9 ± 1.2
ΔN103-OatC	42.0	46.0 ± 0.9	1.1	0.4 ± 0.3
N34-MBP	46.0	114.4 ± 6.8	2.5	ND
MBP	41.9	29.1 ± 1.1	0.7	ND
OatC-S286A	54.3	100.1 ± 1.2	1.9	0.1 ± 0.3
OatC-D376A	54.3	92.4 ± 1.3	1.7	0.2 ± 0.1
OatC-H399A	54.3	92.7 ± 2.3	1.7	0.1 ± 0.4

for ΔN103-OatC, ΔN34-OatC retained 25% of the wild-type activity (Table 2). The latter finding demonstrates that dimerization is not an absolute requirement for enzymatic activity. However, deletion of the first N-terminal amino acids seems to affect protein folding and/or stability, leading to the observed drop in activity. To further evaluate the role of the N-terminal part in providing a dimerization interface, we fused the first 34 amino acids of OatC to MBP of *E. coli*, resulting in the hybrid protein N34-MBP. MBP is a monomeric protein, and in line with previous reports (39, 40), an oligomeric state of 0.7 was determined by gel filtration. By contrast, a quaternary state of 2.5 was found for N34-MBP, providing strong evidence that the first 34 amino acids of OatC are sufficient to mediate protein dimerization even in a different protein context.

Identification of a Catalytically Important Histidine Residue—For OatC, the identification of functionally important residues by visualizing highly conserved residues was impeded by a complete lack of sequence homology between OatC and any other protein in the data bases (22). However, because acetyltransferases of a variety of different protein families comprise catalytically important histidine residues (24, 41–44), we asked whether one of the six histidines found in the primary sequence of OatC (Fig. 2*A*) is involved in enzymatic activity. To address this question, single alanine substitutions were introduced, resulting in OatC variants H31A, H111A, H183A, H267A, H399A, and H456A. All proteins were expressed in *E. coli* BL21(DE3) with a C-terminal hexahistidine tag and were found at comparable levels in the soluble fraction. After isolation by IMAC and size-exclusion chromatography (Fig. 2*B*), equimolar amounts of wild-type and mutant enzymes were analyzed in the spectrophotometric assay. With the exception of OatC-H399A and OatC-H456A, all investigated variants showed enzymatic activity similar to that of the wild-type protein (Fig. 2*C*). Alanine substitution of the most C-terminal histidine residue (H456A) resulted in reduced activity. However, this mutant retained 70% of the wild-type level, excluding an essential role of His-456 in catalysis. By contrast, a complete loss of activity was observed for OatC-H399A, demonstrating that His-399 is critical for enzymatic activity.

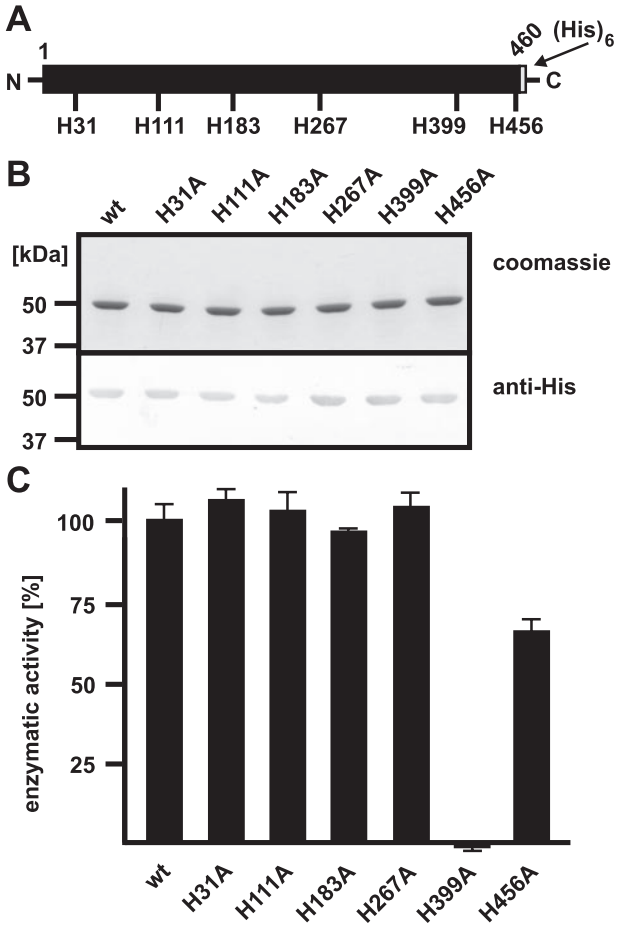

FIGURE 2. **Identification of a catalytic histidine.** *A*, shown is a schematic representation of OatC with a C-terminal hexahistidine tag. The positions of all six histidine residues present in OatC are highlighted. *B*, the single alanine substitutions of the indicated amino acids were introduced into OatC. After expression in *E. coli* BL21(DE3) and affinity purification on Ni^{2+} chelating columns, wild-type (*wt*) and mutant forms were analyzed by 10% SDS-PAGE and Coomassie staining (*upper panel*) and Western blotting using anti-penta-His antibody for detection (*lower panel*). *C*, the enzymatic activities of wild-type and mutant forms were determined in a spectrophotometric assay using equimolar concentrations of each purified enzyme variant. Data represent means ± S.D. of three independent experiments measured in triplicates, and the value obtained for wild-type OatC was set to 100%.

OatC Contains a Nucleophile Elbow Motif—Motif scanning using the MyHits web server (45) revealed a putative nucleophile elbow motif ($GXS^{286}XGG$) in the primary sequence of OatC (Fig. 3*A*). With the consensus sequence Sm-*X*-Nu-*X*-Sm-Sm (where Sm is a small residue, Nu is a nucleophilic residue, and *X* is any residue), the nucleophile elbow represents a structural motif that places a nucleophile at the tip of a sharp turn to allow efficient presentation at the site of attack on the substrate. It is a typical feature of α/β-hydrolase fold enzymes, where the nucleophile (Ser, Cys, or Asp) within the motif is part of a catalytic triad composed of Nu-(Asp/Glu)-His (42, 46–50). Members of the α/β-hydrolase fold family that use serine as a nucleophile can be irreversibly inhibited by the serine-specific reagent phenylmethylsulfonyl fluoride, resulting in sulfonylation of the catalytic serine. Preincubation of purified OatC with phenylmethylsulfonyl fluoride resulted in an almost complete loss of OatC activity (Fig. 3*C*), indicating the presence of a catalytic serine. To determine whether Ser-286 located in the

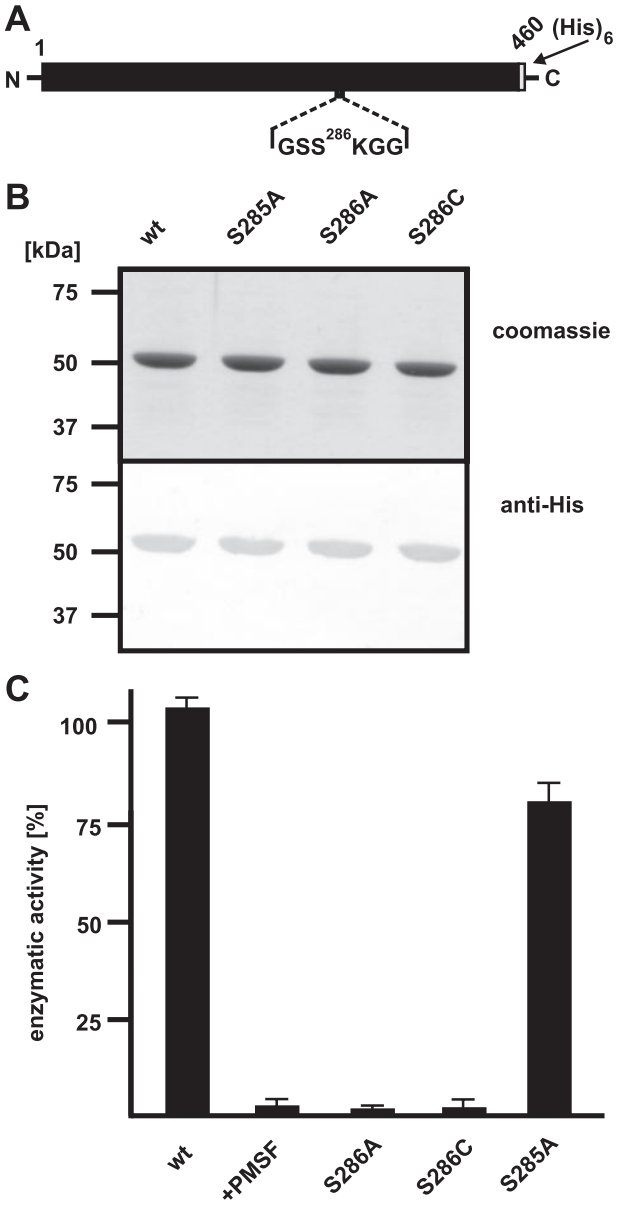

FIGURE 3. **Identification of a nucleophile elbow motif.** *A*, shown is a schematic representation of OatC. The relative positions of the identified nucleophile elbow motif and Ser-286 are indicated. *B*, the single amino acid exchanges S286A, S286C, and S285A were introduced into OatC. After expression in *E. coli* BL21(DE3) and affinity purification on Ni^{2+} chelating columns, wild-type (*wt*) and mutant forms were analyzed by 10% SDS-PAGE and Coomassie staining (*upper panel*) and Western blotting using anti-penta-His antibody for detection (*lower panel*). *C*, the enzymatic activities of wild-type and mutant forms were determined in a spectrophotometric assay using equimolar concentrations of each purified enzyme variant. In parallel, the enzymatic activity of purified wild-type OatC was analyzed in the presence of 10 mM phenylmethylsulfonyl fluoride (+*PMSF*). Data represent means ± S.D. of three independent experiments measured in triplicates, and the value obtained for wild-type OatC was set to 100%.

nucleophile elbow is indeed part of the catalytic site of OatC, this residue was exchanged with alanine and in parallel by cysteine to introduce an alternative nucleophile. Because the nucleophile elbow motif identified in OatC contains a second serine (Ser-285), this residue was also exchanged with alanine. The resulting variants, OatC-S285A, OatC-S286A, and OatC-S286C, were expressed in *E. coli* BL21(DE3) and purified to homogeneity via the C-terminal hexahistidine tag by IMAC and size-exclusion chromatography (Fig. 3*B*). Enzymatic activity was investigated using the spectrophotometric activity assay described above. Whereas OatC-S285A retained 80% of the wild-type activity, alanine substitution of the adjacent Ser-286 resulted in a complete loss of activity (Fig. 3*C*). Replacement by a cysteine did not rescue activity, indicating that OatC activity depends on a serine residue that has to be properly positioned within the nucleophile elbow.

Identification of a Ser-Asp-His Catalytic Triad—To investigate whether His-399 and Ser-286 are part of a catalytic triad, we searched for an acidic residue that would complete the triad. The common order in which the catalytic residues appear in the primary sequence of α/β-hydrolase fold enzymes is Ser-(Asp/Glu)-His (42, 46). Therefore, aspartate and glutamate residues located between Ser-286 and His-399 of OatC (Fig. 4*A*) were likely candidates for the missing catalytic acid. Consequently, we generated OatC variants with the single alanine substitutions E336A, E345A, D359A, D371A, D376A, and E379A that were expressed in *E. coli* BL21(DE3) with a C-terminal hexahistidine tag. The analysis of bacterial lysates by SDS-PAGE combined with either Coomassie staining or Western blot analysis revealed similar expression levels for all variants (Fig. 4*B*). To omit purification of each variant, bacterial lysates were analyzed directly for enzymatic activity. Because the spectrophotometric assay is not compatible with a high background of protein-bound sulfhydryl groups as present in crude lysates, the radioactive incorporation assay was applied. With the single exception of OatC-D376A, all variants showed enzymatic activity similar to that of the wild-type enzyme. To confirm the observation that alanine substitution of Asp-376 results in a complete loss of activity, the respective OatC variant was purified to homogeneity (Fig. 4*D*) and was analyzed in the spectrophotometric assay. In this case, identical amounts of purified enzyme were used for wild-type OatC and OatC-D376A. However, no enzymatic activity was observed for OatC-D376A, demonstrating that Asp-376 is essential for catalysis (Fig. 4*E*) and completes the catalytic triad as the critical acid. It is noteworthy that all three OatC variants in which the catalytic triad was destroyed by the single alanine substitution S286A, D376A, or H399A were found as dimers (see Table 2), suggesting that the introduced mutations did not affect the overall folding of these proteins.

Capsule O-Acetylation in Serogroup C Meningococci Carrying Mutant Forms of oatC—To verify the critical role of Ser-286, Asp-376, and His-399 for OatC activity *in vivo*, we generated a set of isogenic NmC strains carrying different *oatC* variants. Wild-type *oatC* and mutant forms, extended by a sequence encoding a C-terminal hexahistidine tag, were inserted by homologous recombination into the *oatC* locus of NmC strain 2120 (32), thereby replacing the endogenous wild-type gene. OatC expression was monitored in bacterial lysates by Western blot analysis with anti-penta-His antibody. As shown in Fig. 5*A*, all isogenic strains expressed comparable amounts of OatC, whereas no enzyme was detected in the deletion mutant NmC-Δ*oatC* (22), used as a negative control. The capsule *O*-acetylation competence of all strains was monitored in a whole-cell enzyme-linked immunosorbent assay using two capsule-specific antibodies: mAb 924, which binds to α2,9-

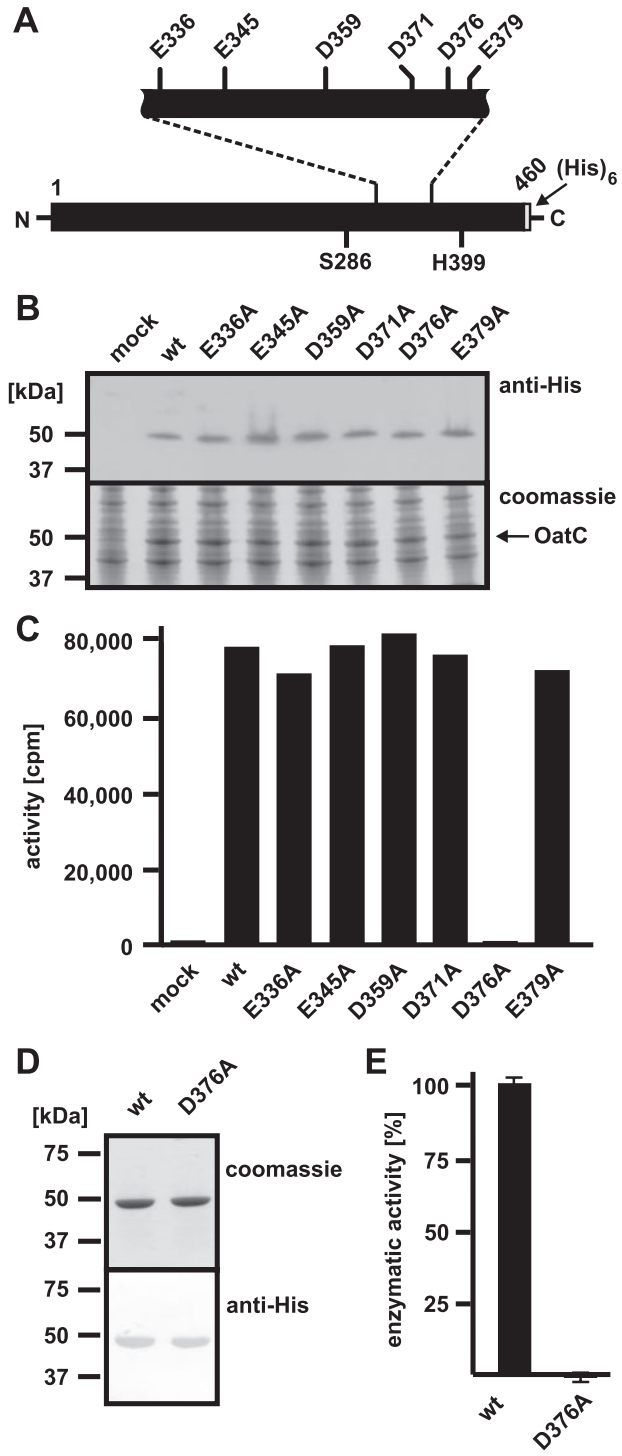

FIGURE 4. **Catalytic acid of OatC.** *A*, shown is a schematic representation of OatC, highlighting the positions of the six aspartate and glutamate residues located between Ser-286 and His-399. *B*, the single amino acid substitutions E336A, E345A, D359A, D371A, D376A, and E379A were introduced into OatC. After expression in *E. coli* BL21(DE3), bacterial lysates of all variants were separated by 10% SDS-PAGE and analyzed by Western blotting using anti-penta-His antibody for detection (*upper panel*) and Coomassie staining (*lower panel*). *C*, the OatC activity of wild-type (*wt*) and mutant forms was determined in bacterial lysates using a radioactive incorporation assay. *D*, wild-type OatC and the variant OatC-D376A were purified by IMAC and size-exclusion chromatography. Isolated proteins were analyzed by 10% SDS-PAGE followed by Coomassie staining (*upper panel*) and Western blot analysis with anti-penta-His antibody (*lower panel*). *E*, the enzymatic activities of the wild-type enzyme and OatC-D376A were determined in a spectrophotometric assay using equimolar concentrations of each purified enzyme variant. Data represent means ± S.D. of three independent experiments measured in triplicates, and the value obtained for wild-type OatC was set to 100%.

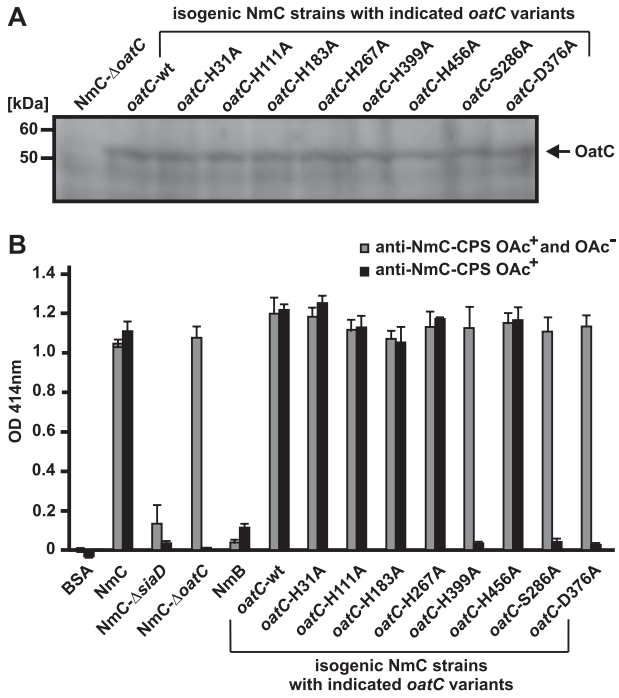

FIGURE 5. **Capsule *O*-acetylation in isogenic NmC strains with different *oatC* variants.** A set of isogenic NmC strains carrying the indicated *oatC* variants was generated by homologous recombination. In all cases, genes extended by a sequence encoding a C-terminal hexahistidine tag were used. *A*, shown are the results from Western blot analysis of bacterial lysates stained with anti-penta-His antibody. As a negative control, an NmC strain lacking *oatC* was used (NmC-Δ*oatC*). *B*, the *O*-acetylation status of the indicated strains was investigated in a whole-cell enzyme-linked immunosorbent assay with mAb 1125 (*black bars*), which is specific for the *O*-acetylated (OAc^+) form of NmC-CPS. Capsule expression was controlled with mAb 924 (*gray bars*), which recognizes NmC-CPS irrespective of the *O*-acetylation status (OAc^+ and OAc^-). As a negative control, bovine serum albumin (*BSA*) was used instead of bacteria. The specificity of the primary antibodies was controlled by using the following strains: a wild-type (*wt*) NmC strain carrying a functional *oatC* gene (NmC), an NmC deletion mutant lacking the polysialyltransferase gene (*siaD*) essential for capsule synthesis (NmC-Δ*siaD*), an NmC deletion mutant lacking *oatC* (NmC-Δ*oatC*), and a serogroup B strain (NmB).

linked polySia irrespective of the presence or absence of *O*-acetylation, and mAb 1125, which recognizes exclusively the *O*-acetylated form of NmC-CPS (32). As shown in Fig. 5*B*, both antibodies gave strong signals on *oatC*-positive NmC wild-type strain 2120. By contrast, complete loss of the OAc-specific signal was observed for the respective null mutant NmC-Δ*oatC*, although capsule expression *per se* was not affected as demonstrated by a strong signal obtained with mAb 924. The specificity of the antibodies used was confirmed by the absence of antibody binding on NmC-Δ*siaD*, which lacks the polysialyltransferase essential for capsule synthesis (51), and on NmB, which expresses a polySia capsule composed of α2,8-linked Neu5Ac (Fig. 5*B*). Analysis of the newly generated isogenic NmC strains revealed capsule *O*-acetylation in those strains that express an enzyme variant that was proved active *in vitro* (here, variants H31A, H111A, H183A, H267A, and H456A are shown as an example). By contrast, no signal with OAc-specific antibody mAb 1125 was obtained for mutant strains comprising amino acid substitution H399A, S286A, or D376A. The lack

of mAb 1125 binding was not due to reduced capsule expression because in all cases strong signals were obtained with mAb 924. Thus, in line with the *in vitro* findings, no enzymatic activity was found for OatC-H399A, OatC-S286A, and OatC-D376A *in vivo*. Together, these results confirm the presence of a catalytic triad composed of Ser-286, Asp-376, and His-399.

Secondary Fold Prediction—Members of the α/β-hydrolase fold family share a conserved eight-stranded mostly parallel β-sheet, flanked on both sides by helices. The presence of this fold might become evident from the arrangement of the secondary structure elements. As shown in supplemental Fig. S2, we propose a topology diagram for OatC that is based on secondary structure prediction (52). Whereas the first helix (αA) and the first three β-strands (β1–3) could not be unequivocally assigned, secondary structure elements predicted for amino acids 259–422 show the typical topology of a "canonical" α/β-fold. The positions of the catalytic residues Ser-286, Asp-376, and His-399 after strands β5, β7, and β8, respectively, also matched the commonly found location of the catalytic triad (46, 53, 54).

Catalytic Mechanism of OatC—Most α/β-hydrolase fold enzymes act via a double-displacement (ping-pong) mechanism (42, 55–58). In the case of acetyltransferases belonging to the α/β-hydrolase fold family, this involves the formation of a covalent acetyl-enzyme intermediate that occurs by transfer of the acetyl group from acetyl-CoA onto the serine residue located in the nucleophile elbow (47, 58, 59). In the second half of the reaction, the acetyl group is further transferred onto the acceptor substrate.

To gain the first insight into the catalytic mechanism of OatC, we monitored the enzyme reaction for the occurrence of a stable acetyl-enzyme intermediate. Therefore, purified OatC was incubated with [^{14}C]acetyl-CoA, and the reaction mixture was analyzed before and after addition of the acceptor substrate by SDS-PAGE followed by autoradiography (Fig. 6*A*) or combined Alcian blue/silver staining as a loading control (Fig. 6*B*). For wild-type OatC, a radioactively labeled protein band migrating with the expected molecular mass of 54.3 kDa was observed, indicating the appearance of an acetyl-enzyme intermediate. Upon addition of NmC-CPS, this intermediate collapsed, and radiolabeled NmC-CPS appeared (Fig. 6*A*), which is consistent with the transfer of the radioactive acetyl group from the enzyme onto the acceptor substrate. By contrast, no acetyl-enzyme intermediate was detected for OatC-S286A, suggesting that the hydroxyl group of Ser-286 acts as acceptor site for the acetyl group. In the case of the site-specific variant D376A, the appearance of an acetyl-enzyme intermediate could be detected, although the amount of radiolabeled enzyme was lower than for the wild-type enzyme. However, addition of the acceptor substrate did not result in a collapse of the intermediate, and accordingly, no transfer onto NmC-CPS was detected (Fig. 6*A*). This result indicates that Asp-376 is dispensable for the first half of the reaction but essential for the second half. Similar to the S286A exchange, alanine substitution of His-399 abolished the formation of an acetyl-enzyme intermediate, suggesting an important role of His-399 during transfer of the acetyl group onto the enzyme. Thus, analysis of wild-type and mutant OatC demonstrated that *O*-acetylation of NmC-CPS proceeds via a covalent acetyl-enzyme intermediate that involves Ser-286.

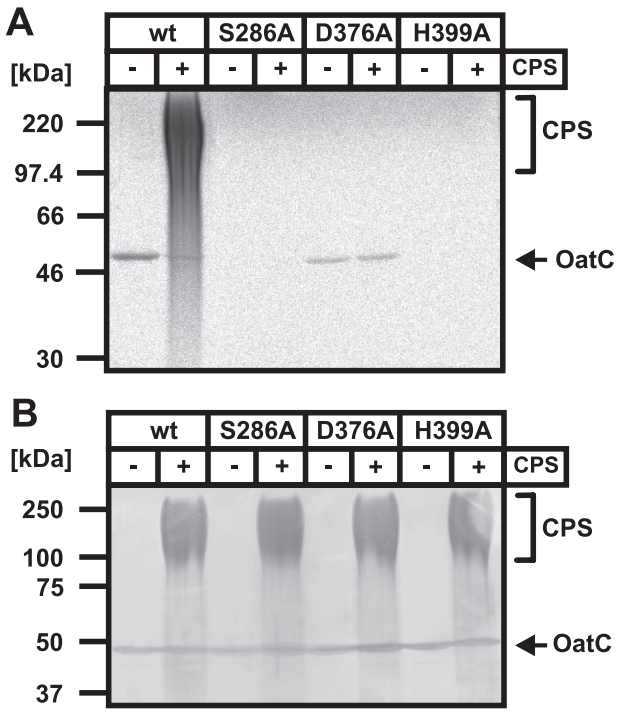

FIGURE 6. **Identification of an acetyl-enzyme intermediate.** Purified wild-type OatC and variants containing the single amino acid exchanges S286A, D376A, and H399A were incubated with [^{14}C]acetyl-CoA. Before (−) and after (+) addition of the acceptor substrate NmC-CPS, aliquots of the reaction mixture were analyzed by 10% SDS-PAGE followed by phosphorimaging to visualize radioactively labeled enzyme and acceptor substrate (*A*) or combined Alcian blue/silver staining to control identical loading of enzyme and NmC-CPS (*B*). Bands representing OatC and NmC-CPS are indicated on the *right*.

DISCUSSION

In this study, we performed the first purification and biochemical characterization of OatC, the capsule *O*-acetyltransferase of serogroup C meningococci. The enzyme proved to be highly specific for polySia joined by α2,9-linkages and did not act on α2,8-linked polySia or on sialic acid-containing heteropolymers such as CPS of serogroups W-135 and Y. Moreover, free Neu5Ac and CMP-Neu5Ac were not modified by OatC, indicating that *in vivo O*-acetylation of NmC-CPS occurs exclusively at the polymer level. Similar observations have been made for NeuO, a prophage-encoded sialate *O*-acetyltransferase found in *E. coli* K1 strains that harbor the CUS-3 phage (25). NeuO catalyzes the *O*-acetylation of K1-CPS, and the enzyme acts exclusively on α2,8-linked polySia with at least 14 sialic acid residues (26, 60).

Despite the functional similarity between NeuO and OatC, our data presented in this study point out that the two enzymes evolved independently and belong to structurally diverse protein families. As shown recently by homology modeling and site-directed mutagenesis studies, NeuO belongs to the hexapeptide repeat family of acyltransferases (26). Members of this protein family contain a unique triangular left-handed β-helix fold with a protruding loop that harbors the catalytic histidine residue. They assemble into catalytic trimers in which the protruding loop of one subunit embraces the left-handed

β-helix domain of the adjacent subunit, resulting in three identical active sites at the interfaces between the subunits (24, 61–63). In the case of NeuO, hexamers were found in solution, which might be dimers of trimers (26).

In contrast to NeuO, the primary sequence of OatC is devoid of hexapeptide repeats. Moreover, BLAST search analyses did not reveal significant sequence similarities between OatC and any other protein (22), impeding the identification of phylogenetic relationships. However, by combining bioinformatic approaches (motif scanning and secondary structure prediction), site-directed mutagenesis, and biochemical analyses, we have now identified several features that characterize OatC as a new member of the α/β-hydrolase fold family of proteins. The α/β-hydrolase fold is a versatile and widespread protein architecture that is found in enzymes with diverse functions such as proteases, lipases, esterases, dehalogenases, haloperoxidases, lyases, and epoxide hydrolases (42, 50, 64–70). The various members of this large superfamily usually lack significant amino acid sequence similarities but share a common fold that gave the family the name. The canonical α/β-hydrolase fold is composed of an eight-stranded mainly parallel β-structure that arranges in a twisted β-sheet, flanked on both sides by helices (42, 46). The fold provides a stable scaffold for a catalytic triad consisting of a nucleophile (serine, cysteine, or aspartate), an acid (aspartate or glutamate), and a histidine, with the nucleophile located at the tip of a sharp turn, the so-called "nucleophile elbow." However, many variations from the canonical fold have been observed mainly due to the absence of particular strands or helices and/or insertions that can range from a few amino acids to entire domains. Based on the large number of solved crystal structures, the essential features to identify the fold are the presence of at least five parallel β-strands, the order of the catalytic triad (nucleophile-acid-histidine), and a nucleophile elbow at the top of canonical strand β5 (54).

Although a three-dimensional structure is required to unequivocally confirm the presence of a particular fold, our data provide strong evidence that OatC adopts an α/β-hydrolase fold. A series of single amino acid exchanges led to the identification of a catalytic triad composed of Ser-286, Asp-376, and His-399. Ser-286 was found to be part of a typical nucleophile elbow motif (G*X*S*X*GG), indicating that similar to other α/β-hydrolase fold proteins, the nucleophile is located in a tight turn to expose the hydroxyl group of Ser-286 to nucleophilic attack of the incoming substrate. Moreover, secondary structure prediction suggested for OatC a pattern of β-strands and α-helices that matches the topology of the canonical α/β-hydrolase fold (42, 46, 53, 54). In the proposed topology model, the catalytic residues are located after strands β5, β7, and β8 for Ser-286, Asp-376, and His-399, respectively, which is consistent with the common positioning of the catalytic triad in α/β-hydrolase fold enzymes.

Members of the α/β-hydrolase fold family catalyze a variety of transformations. However, for almost all enzymes, a double-displacement (ping-pong) kinetic mechanism is described that involves a covalent substrate-enzyme intermediate with the substrate linked to the nucleophile of the catalytic triad (42, 58, 66, 71–76). In line with this, a stable acetyl-enzyme intermediate was observed for OatC that crucially depended on the presence of Ser-286. In addition, a CoA burst was observed in the absence of polySia (data not shown). This indicates that the first substrate is released before binding of the second substrate, which confirms a ping-pong mechanism. On the basis of our data obtained for wild-type OatC and mutant forms, we propose the following catalytic mechanism (Fig. 7). The carboxyl group of Asp-376 forms a salt bridge with the N-δ1 of His-399, allowing the N-ϵ2 of the imidazole ring to abstract a proton from Ser-286. Thereby, the nucleophilicity of the hydroxyl group of Ser-286 is increased, facilitating the attack of the carbonyl carbon of acetyl-CoA. The resulting tetrahedral transition state subsequently collapses to an acetyl-enzyme intermediate with the acetyl moiety covalently attached to Ser-286. During the acetylation step, the imidazole group of His-399 transfers the proton of the serine hydroxyl to the sulfhydryl leaving group, and free CoA is released. The second half-reaction is also assisted by His-399 through acid/base catalysis and starts with the abstraction of a proton from the C-7 (or C-8) hydroxyl group of the acceptor polysaccharide. This enables the nucleophilic attack of the carbonyl carbon of the acetyl group in the acetyl-enzyme intermediate, resulting in a second tetrahedral transition state. Subsequent decomposition of the tetrahedral intermediate leads to release of the second reaction product, *i.e.* *O*-acetylated NmC-CPS. In this last step, Ser-286 is returned to its protonated state, which reinitializes OatC for a new catalytic cycle.

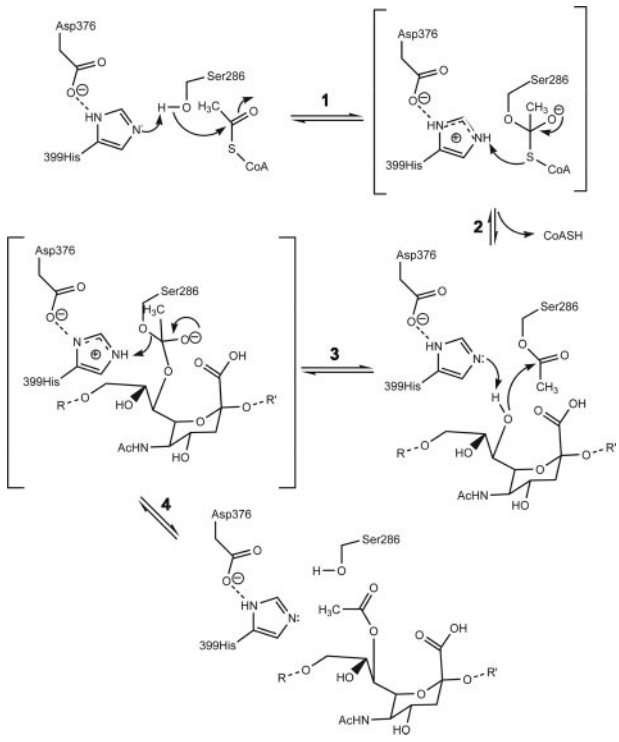

FIGURE 7. **Proposed reaction mechanism of the OatC-catalyzed *O*-acetylation of NmC-CPS.** The reaction proceeds through the following steps: *step 1*, nucleophilic attack of the carbonyl group of acetyl-CoA by Ser-286 and formation of the first tetrahedral intermediate; *step 2*, decomposition of the tetrahedral intermediate, release of free CoA, and formation of an acetyl-enzyme intermediate; *step 3*, nucleophilic attack by the C-7 (or C-8) hydroxyl group of the acceptor polysaccharide and formation of a second tetrahedral intermediate; and *step 4*, release of the second product (*O*-acetylated NmC-CPS) and regeneration of free enzyme.

Further studies are required to investigate whether OatC transfers acetyl groups to both the C-7 and C-8 hydroxyl groups. Using ^{13}C NMR spectroscopy, Bhattacharjee *et al.* (14) detected di-*O*-acetylated residues (Neu5,7,8Ac_3) in NmC-CPS, whereas no evidence for di-*O*-acetylated sialic acids was found by Lemercinier and Jones (17). For NmC-CPS containing only mono-*O*-acetylated residues, non-enzymatic migration of acetyl groups was observed. In freshly prepared CPS, *O*-acetylation was found predominantly at O-8, whereas after storage at room temperature, the majority of acetyl groups were shifted to O-7 (17, 77, 78). Therefore, OatC might be selective for O-8, and acetylation of O-7 might occur exclusively by non-enzymatic migration.

In agreement with the proposed critical roles of the catalytic residues of OatC, single alanine substitutions of Ser-286, Asp-376, and His-399 completely abolished OatC activity *in vitro* and *in vivo*. Replacement of either His-399 or Ser-286 already abrogated the ability to form a covalent acetyl-enzyme intermediate, which is consistent with the proposed crucial function of His-399 as a general base catalyst and the central role of Ser-286 as an attachment site for the acetyl group. In contrast to OatC-H399A and OatC-S286A, OatC-D376A was still able to form a limited amount of acetyl-enzyme intermediate, indicating that Asp-376 is dispensable during the first half-reaction but essentially required for catalysis of the second half-reaction. This finding is in accordance with the less pronounced role of the catalytic acid observed in many α/β-hydrolase fold enzymes. In several cases, alanine substitution of the catalytic acid was even compatible with enzyme catalysis, and considerably high levels of activity were observed (66, 76, 79).

We conclude that OatC adopts an α/β-hydrolase fold structure with a Ser-Asp-His catalytic triad that forms the molecular basis for *O*-acetylation of NmC-CPS by a ping-pong mechanism. This clearly distinguishes OatC from all other bacterial sialate *O*-acetyltransferases known so far, which all belong to the hexapeptide repeat family: (i) NeuO, responsible for *O*-acetylation of the *E. coli* K1 capsule; (ii) OatWY, involved in *O*-acetylation of sialic acids within the galactose- and glucose-containing heteropolymeric polySia capsules of serogroup W-135 and Y meningococci, respectively (22); (iii) NeuD of group B streptococci, required for *O*-acetylation of terminal α2,3-linked sialic acids capping CPS (28); and (iv) NeuD of *C. jejuni*, catalyzing the *O*-acetylation of terminal α2,8-linked sialic acids of the lipo-oligosaccharide (27). Compared with α/β-hydrolase fold enzymes, members of the hexapeptide repeat family not only adopt an entirely different structural fold, the left-handed β-helix fold, but also act by a different kinetic mechanism. Although they possess a strictly conserved catalytic histidine, this residue is not part of a catalytic triad, and kinetic data obtained for this enzyme family revealed a sequential kinetic mechanism (24, 62, 80, 81).

The observed striking differences between OatC and other bacterial sialic acid-modifying *O*-acetyltransferases indicate that the enzymatic function of sialate *O*-acetylation evolved independently on two distinct structural frameworks by convergent evolution. Whereas the hexapeptide repeat family of acyltransferases is restricted to microorganisms, α/β-hydrolase fold enzymes are present in all kingdoms of life. Interestingly, the α/β-hydrolase fold is also used as the structural basis for viral sialic acid-specific acetylesterases, the receptor-destroying enzymes of influenza C and coronaviruses that use *O*-acetylated sialic acids as host receptor structures (82, 83). Thus, this fold appears to be a likely candidate for the structural framework of eukaryotic sialate *O*-acetyltransferases, for which the genetic and structural bases are still unknown. Attempts to clone the respective genes, *e.g.* by expression cloning, failed, and purification and characterization of the enzymatic activities proved to be challenging (84–88). The new insight in the evolution of sialate *O*-acetyltransferases obtained in bacteria may help to identify similar enzymes in eukaryotes by searching for genes encoding potential α/β-hydrolase fold proteins with unknown function.

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