Identification of Arg-12 in the active site of *Escherichia coli* K1 CMP-sialic acid synthetase

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Escherichia coli K1 CMP-sialic acid synthetase catalyses the synthesis of CMP-sialic acid from CTP and sialic acid. The active site of the 418 amino acid *E. coli* enzyme was localized to its N-terminal half. The bacterial CMP-sialic acid synthetase enzymes have a conserved motif, IAIIPARXXSKGLXXKN, at their N-termini. Several basic residues have been identified at or near the active site of the *E. coli* enzyme by chemical modification and site-directed mutagenesis. Only one of the lysines in the N-terminal motif, Lys-21, appears to be essential for activity. Mutation of Lys-21 in the N-terminal motif results in an inactive enzyme. Furthermore, Arg-12 of the N-terminal motif appears to

be an active-site residue, based on the following evidence. Substituting Arg-12 with glycine or alanine resulted in inactive enzymes, indicating that this residue is required for enzymic activity. The Arg-12 → Lys mutant was partially active, demonstrating that a positive charge is required at this site. Steady-state kinetic analysis reveals changes in k_{cat} , K_m and K_s for CTP, which implicates Arg-12 in catalysis and substrate binding.

Key words: *N*-acetylneuraminic acid, enzyme mutagenesis, sugar activation.

INTRODUCTION

In bacteria and vertebrates *N*-acetylneuraminic acid (NeuNAc) is transferred to glycoconjugates from the sugar nucleotide substrate CMP-NeuNAc by sialyltransferases. The enzyme that synthesizes this common sugar nucleotide substrate, *N*-acetylneuraminic acid cytidyltransferase (EC 2.7.7.43), catalyses the formation of CMP-NeuNAc in the following reaction:

This enzyme is prevalent in vertebrates and in bacteria capable of sialylating extracellular carbohydrates [1,2]. The enzyme has been purified from bacterial and vertebrate sources [3–9]. A functional enzyme has been demonstrated in *Escherichia coli* [3], *Neisseria meningitidis* [10], *Haemophilus ducreyi* [5], *Campylobacter coli* [11] and *Streptococcus* Group B [12]. The gene encoding CMP-NeuNAc synthetase has been identified in these bacteria. The existence of a CMP-NeuNAc synthetase in *Haemophilus influenzae* has been inferred from its genome sequence. The *E. coli* K1 enzyme catalyses the nucleophilic attack of the anomeric oxygen of β -NeuNAc on the α -phosphate of CTP [13]. This mechanism is similar to that proposed for the 2-oxo-3-deoxy-manno-octonic acid cytidylmonophosphoryltransferase (CMP-KDO synthetase) [14].

As part of a study of the biosynthetic pathway for the synthesis of bacterial polysialic acid capsules, our laboratory has identified and characterized the CMP-NeuNAc synthetase gene (*neuA*) in *E. coli* K1 [4,15]. Our objective in the present studies was to define residues in the active site and their role in catalysis. Chemical modification experiments and sequence comparisons were used to identify a putative catalytic domain of the *E. coli* CMP-sialic acid synthetase. In the present report we identify two

basic residues by site-directed mutagenesis which are important for catalytic activity of *E. coli* K1 CMP-sialic acid synthetase.

MATERIALS AND METHODS

Materials

Nickel-nitrilotriacetic acid–agarose and plasmid DNA prep kits were obtained from QIAGEN. Altered sites II *in vitro* mutagenesis system, the fmol DNA sequencing kit, *Taq* polymerase and all DNA-modifying enzymes were purchased from Promega, except for *Nde*I and *Vent* polymerase, which were obtained from New England Biolabs. Radiolabelled materials were purchased from Amersham. CTP (Type VII), NeuNAc (Type VI or VIII), CMP-NeuNAc and Bicine were from Sigma. Pefabloc [4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride] and complete protease inhibitor cocktail were from Boehringer Mannheim Biochemical. Oligonucleotide synthesis was performed by the Facility for Biotechnology Resources, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, U.S.A.

Bacterial strains and vectors

Bacterial strain DH5 α was used for expression of wild-type and mutant synthetase enzymes. The mismatch repair-deficient and tetracycline-resistant strain BMH 71-18 *mutS* [*supE thi-1 Δ (lac-proAB) F'(proAB⁺ lacI^q Δ M15) mutS::Tn10]* (Clontech) was used for selection and propagation of mutagenized single-strand DNA, instead of the ES1301 *mutS* strain supplied in the altered sites II *in vitro* mutagenesis system. Vectors pSP72 and pALTER-Ex2 were purchased from Promega, whereas vector pET-27b(+) was obtained from Novagen. The plasmid pWA1, which contains

Abbreviations used: DTT, dithiothreitol; HSV, herpes simplex virus epitope; HIS, hexahistidine tag; NeuNAc, *N*-acetylneuraminic acid; PLP, pyridoxal phosphate; oCTP, periodate-oxidized CTP; CMP-KDO, CMP-2-oxo-3-deoxy-manno-octonic acid.

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the *neuA* gene, has been described previously [15]. Host strains do not have background CMP-NeuNAc synthetase activity.

Construction of the pWS313 expression plasmid

All primer sequences are presented 5' → 3' with mutagenic sequence(s) in lower case. The *neuA* gene was amplified from 5 ng of the plasmid pWA1 using Vent polymerase in a 100 µl reaction, as recommended by the manufacturer. Two reactions were performed to introduce a unique *EcoRI* site by mutating codon 245 from TTT to TTC, both of which encode phenylalanine. One reaction contained the primers *Bam*HI-*neuA* (cgcgatccATGAGAACAAAAATTATTGCG) and *Eco*RI-R (5'-ATCgAATTCATTTCTGTTCTCATTTG), while the other had primers *neuA*-*Xho*I (cgctcgcagTTTAACAATCTCCGCTATTTC) and *Eco*RI-F (AATGAATTCGATAGTGTAAGTGATA-TAACT). The resultant PCR products were digested with the appropriate restriction enzymes and ligated into the *Bam*HI and *Xho*I sites of pET-27b(+), which resulted in the herpes simplex virus epitope and hexahistidine-tagged *neuA*-*HSV*-*HIS* gene. This gene was then amplified as above with primers *Bam*HI-*neuA* and *HIS*-*Nsi*I (atcatgcatTCAGTGGTGGTGGTGGT) and cloned into the *Bam*HI and *Nsi*I sites of plasmid pALTER-Ex2. Before cloning into pALTER-Ex2, the *Eco*RI site in the multicloning region of this vector was destroyed by digesting with *Eco*RI, filling in the overhang with Vent polymerase, and then re-ligating the blunt ends. The *neuA*-*HSV*-*HIS*/pALTER-Ex2 expression construct, named pWS306, was then mutated to chloramphenicol resistance using the altered sites II *in vitro* mutagenesis system, except that strain BMH 71-18 *mutS* was used to propagate the synthesized DNA strand instead of the ES1301 *mutS* strain. The resulting tetracycline- and chloramphenicol-resistant expression plasmid pWS313 was then subjected to DNA sequencing to ensure that the entire *neuA*-*HSV*-*HIS* sequence was correct.

Primers used in site-directed mutagenesis by recombinant PCR

Outer primers

*Bam*HI-*neuA*: 5'-CCGGATCCATGAGAACAAAAATTATTGCG

neuA-811-R: 5'-CCCAATAATCAAACAGCGAGTGTCC

Mutagenic primers

R12A-F: 5'-CCAGCCgcTAGTGGATCTAAAGGGTTG

R12A-R: 5'-TCCACTAgcGGCTGGAATTATCGCAAT

R12K-F: 5'-CCAGCCaaaAGTGGATCTAAAGGGTTG

R12K-R1: 5'-AGATCCACTtttGGCTGGAATTATCGCAAT

K16A-F: 5'-AGTGGATCTgctGGGTTGAGAAATAAAAAT-GC

K16A-R: 5'-TCTCAACCCagcAGATCCACTACGGGC

K21A-F: 5'-TTGAGAAATgcAAATGCTTTGATGCTGATAGA

K21A-R: 5'-CAAAGCATTTgcATTTCTCAACCCTTTAGA

K21R-F: 5'-TTGAGAAATAgAAATGCTTTGATGCTGATAGA

K21R-R: 5'-CAAAGCATTTcTATTTCTCAACCCTTTAGATC

Site-directed mutagenesis

Mutagenesis was performed by the recombinant PCR method [16] with the primers listed above. In this method two sets of reactions were initially performed per mutant, with one reaction

containing the forward mutagenic primer and the outer reverse primer, while the second reaction contained the reverse mutagenic primer and the forward outer primer. These two reactions were performed in a 50 µl volume containing 5–10 ng of pWS313 as a template, 200 pmol of each primer, and Vent polymerase, as described above. The resulting products were electrophoretically purified in a 2.5 % low-melting-point agarose gel, excised, and melted at 65 °C with a 2-fold dilution of water (v/w). A second PCR reaction was then performed with *Taq* polymerase, 50 pmol each of the outer primers and 1–5 µl of the appropriate forward and reverse PCR fragment solution in a final volume of 100 µl. The resulting products were ethanol-precipitated, digested with *Bam*HI and *Nde*I, and ligated into these sites in pWS313. Transformants were selected on Luria broth/tetracycline/chloramphenicol plates and the mutant plasmids were screened by DNA sequence analysis over the ligated region. Transformants containing plasmids that encode the R12A (pWS348), R12K (pWS370), K16A (pWS331), K21A (pWS336) or K21R (pWS375) mutations were identified (using one-letter symbols for amino acids).

Random mutagenesis at codon 12 was performed as previously described [17].

Enzyme activity assay

The activity of the purified synthetases was determined by incubating various amounts of these enzymes in a 250 µl reaction solution containing 0.2 M Bicine, pH 9.0, 40 mM MgCl₂, 0.2 mM dithiothreitol (DTT), 5 mM CTP and 5 mM NeuNAc. The reactions were incubated for 30 min to 5 h at 37 °C and the quantity of CMP-NeuNAc formed was determined by the modified thiobarbituric acid assay [3,4].

Protein expression and purification

Native CMP-NeuNAc synthetase was expressed and purified as previously described [17]. All epitope-tagged CMP-NeuNAc synthetase (*neuA*-*HSV*-*HIS*) enzymes were expressed by growing precultures of transformants in LB/tetracycline/chloramphenicol/0.2 % glucose overnight at 37 °C. The precultures were diluted 1:2000 in 1.5 litres of Luria broth/tetracycline/chloramphenicol and grown overnight at 37 °C. The cell pellet harvested from 9 litres of culture was resuspended at 4 °C in 80 ml of buffer A (50 mM Bicine/0.1 M NaCl/5 mM 2-mercaptoethanol, pH 8.0), containing 1 mM Pefabloc and complete protease inhibitor cocktail (according to the manufacturer's directions). Cells were lysed by two passages through a French pressure cell at 12000 lb/in² (1 lb/in² = 6.9 kPa) and the cellular debris was pelleted at 12000 g for 15 min. A cytoplasmic extract was obtained by centrifuging this supernatant at 160000 g (*r*_{max}) for 1 h to remove membrane fragments. The cytoplasmic extract (70–75 ml) was applied to a 50 ml DEAE-Sephacel (Pharmacia Biotech) column equilibrated with buffer A containing 0.1 mM Pefabloc. The column was washed extensively with the same buffer to remove unbound protein detectable at 280 nm. The column was then eluted with buffer B (50 mM Bicine/0.3 M NaCl/5 mM 2-mercaptoethanol, pH 8.0) containing 0.1 mM Pefabloc. The resulting eluate was pooled and applied to a 10 ml nickel-nitrilotriacetic acid-agarose column equilibrated with buffer B containing 0.1 mM Pefabloc. The column was washed and the enzyme was eluted with a 0–0.3 M imidazole gradient in buffer A. Peak fractions eluted with the gradient were pooled and precipitated with 65 % ammonium sulphate overnight. The precipitate was dissolved in 5–6 ml of buffer A and dialysed extensively against buffer A without 2-mercaptoethanol. The

protein concentration was determined using a molar absorption coefficient at 278 nm of $54.854 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [17].

Chemical modification of untagged CMP-NeuNAc synthetase

The purified and untagged CMP-NeuNAc synthetase was chemically modified with 0.5 mM phenylglyoxal in buffer C (50 mM Bicine/1 mM DTT/20 mM MgCl_2 , pH 9.0). This solution was incubated at room temperature and 10 μl aliquots were removed periodically and quenched with 40 μl of 1 M arginine. Inactivation of the enzyme was assessed by determining the activity of the quenched solutions (see below).

Pyridoxal phosphate (PLP) inactivation was performed by incubating 256 μg of enzyme in buffer C containing 0.7–2 mM PLP at room temperature. Periodically, 10 μl aliquots were removed and incubated with 10 μl of 0.1 M sodium borohydride at room temperature for 15 min to reduce the Schiff's base formed during the inactivation reaction. Aliquots of the inactivation reaction were quenched with 75 μl of 1 M glycine and the activity of the modified enzyme was assessed. To determine the stoichiometry of 2 mM PLP, inactivation reaction mixtures were reduced with 20 mM sodium borohydride for 15 min and the reaction was quenched with 1 M glycine. After dialysis against buffer C, the concentration of PLP was determined using the absorption coefficient at 327 nm of $1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Inactivation with 2 mM periodate-oxidized CTP (oCTP) was performed in buffer C containing 2 mM oCTP and 20 mM sodium cyanoborohydride. Periodically, 25 μl aliquots were removed and quenched with 75 μl of 1 M glycine before determination of the enzymic activity. For substrate protection experiments, all of the above inactivation reactions were done in the presence of 20 mM CTP.

Kinetic analysis

For the kinetic analysis, enzymic reactions were performed in 250 μl solutions containing 0.2 M Bicine, pH 9.0, 20 mM MgCl_2 and 0.2 mM DTT, with various amounts of CTP (0.2–5 mM) and NeuNAc (6–18 mM). These reactions were incubated at 37 °C for 30–60 min and the amount of product formed was determined as above, except that 100 μl of 0.25 M NaIO_4 solution [3] was used. In preliminary experiments activity was measured at several time points per assay from 0–60 min to validate the appropriateness of the single-point assay. Rates at a given substrate concentration are linear with enzyme concentration. Each kinetic experiment was done in duplicate and repeated at least three times. All results were fitted to eqn. (1) by Hanes–Woolf analysis using the program GraphPad PRISM. The K_m for NeuNAc and the V_{max} were then determined by a secondary graph, plotting the reciprocals of the concentration of NeuNAc versus the reciprocal of the apparent V_{max} .

$$V = \frac{V_{\text{max}}}{1 + \frac{K_m(\text{CTP})}{[\text{CTP}]} + \frac{K_m(\text{NeuNAc})}{[\text{NeuNAc}]} + \frac{K_s(\text{CTP})K_m(\text{NeuNAc})}{[\text{CTP}][\text{NeuNAc}]}}$$

Thermal-stability studies

The thermal stability of purified enzyme was conveniently measured by light scattering at 300 nm, since *E. coli* CMP-sialic acid synthetase readily aggregates on denaturation [17]. Measurements were made over the temperature range 20–70 °C, in 1 °C increments, with a 1 min temperature equilibration time before reading of the absorbance using a Hewlett-Packard 8452A spectrophotometer system equipped with a 8909A temperature

controller. The system software was used to record the results and calculate the temperature at which half of the enzyme was aggregated ($T_{1/2}$). Measurements were done in duplicate at 150 μg of protein/ml in 50 mM Bicine/0.1 M NaCl, pH 8.0.

RESULTS AND DISCUSSION

Multiple sequence alignments of various bacterial CMP-NeuNAc synthetases

A multiple sequence alignment [18] was performed on six bacterial CMP-NeuNAc synthetase enzymes to identify conserved residues which may be present in the active site. All of the similarity between this group of enzymes and the *E. coli* CMP-NeuNAc synthetase is in the first 229 residues, suggesting that the active site resides in this region. This is emphasized by the fact that several of these bacterial enzymes are only 228 amino acids in length, and yet are fully functional [5,19]. Enzymes with nucleotide substrates frequently have essential basic residues at their active site [20,21]. This multiple alignment identified only six conserved lysine and arginine residues. Three of these residues, namely R12, K16 and K21, reside within the first 21 amino acids of the *E. coli* K1 enzyme as part of a conserved motif, IAIIPARXXSKGLXXKN, among bacterial CMP-NeuNAc synthetase enzymes (Figure 1). This motif has also been observed in the N-terminal sequence of the murine pituitary CMP-NeuNAc synthetase [9]. Comparison of these sequences with the CMP-KDO synthetase, which catalyses a reaction similar to that catalysed by CMP-NeuNAc synthetases, showed that residues R12 and K21 are the only conserved basic residues across both groups. A recently published crystal structure of CMP-KDO synthetase implicates N-terminal arginine and lysine residues at the active site of this enzyme [22].

Chemical modification of CMP-NeuNAc synthetase

To test for the presence of important lysine residues, the *E. coli* synthetase was treated with two aldehyde reagents. The substrate analogue oCTP was used as an affinity label to modify potential active-site residues. The reagent inactivates the purified *E. coli* CMP-NeuNAc synthetase (Figure 2). Periodate-oxidized nucleotides have been used to modify lysine, cysteine and tyrosine residues in other enzymes. That the inactivation reaction with *E. coli* CMP-NeuNAc synthetase involves a lysine residue was indicated by the reversibility of the inactivation by glycine and

Figure 1 Sequence alignment of the conserved region between CMP-NeuNAc and CMP-KDO synthetases

The first 60 amino acids are shown and the identical residues are shaded. Numbering is based on the *E. coli* K1 NeuA sequence. The first six lines contain CMP-NeuNAc synthetases from *E. coli* K1 (*E. coli*), group B *Streptococcus agalactiae* (*S. agal.*), *H. ducrey* (*H. ducrey*), *N. meningitidis* (*N. men.*), *H. influenzae* (*H. inf.*), and *C. coli*. The remaining CMP-KDO synthetase sequences are from *E. coli* K5 (*kpsU*), *E. coli* (*kdsB*) and *Chlamydia trachomatis* (*C. trach. kdsB*). The boxed residues are a conserved motif among CMP-sialic acid synthetase enzymes. The asterisks refer to conserved basic residues at the N-terminus.

Figure 2 Inactivation of CMP-NeuNAc synthetase with PLP and oCTP

CMP-NeuNAc synthetase was incubated as described in the Materials and methods section in (A) with PLP (▼) and PLP+CTP (□). In (B) enzyme was incubated with 4.5 mM oCTP/10 mM sodium cyanoborohydride in the presence (□) or absence (▼) of 18 mM CTP. Samples were assayed as described in the Materials and methods section.

the irreversible inactivation in the presence of sodium cyanoborohydride. The enzyme could be protected against inactivation by the substrate CTP, suggesting a potential lysine at the active site [20]. In similar experiments, the aldehyde-containing reagent PLP rapidly inactivated the enzyme, in a manner that was reversed by the addition of glycine, but was irreversible after sodium borohydride reduction. This result demonstrates that PLP probably modifies lysine residues through formation of a Schiff's base [20]. After a loss of 90% of enzyme activity during a 30 min incubation, on average 5 moles of PLP per mole of enzyme, based on the subunit molecular mass of the enzyme, was incorporated, corresponding to only five lysine residues. This inactivation was inhibited by CTP (Figure 2), supporting the notion that lysine residues are located at or near the active site.

In addition to lysine, arginine often plays a key role in the catalytic site of nucleotide-binding enzymes [20,21]. Thus, as

expected from this premise, *E. coli* CMP-NeuNAc synthetase was inactivated by the arginine-specific reagent phenylglyoxal. Arginine residues also appear to reside near the active site, since CTP protects the enzyme against inactivation. These results suggest that both arginine and lysine residues may play some role in the catalysis of CMP-NeuNAc formation and support the notion that conserved basic residues in the motif IAIPARXX-SKGLXXKN are catalytically important and are therefore targets for site-directed mutagenesis.

Since R12 is a candidate for a catalytically important residue, codon 12 was randomly mutated using PCR mutagenesis. Mutants were screened for their ability to complement the synthetase-negative EV-5 strain on antiserum agar plates. The mutated *neuA* DNA from several capsule-negative and capsule-positive isolates was sequenced to determine the nature of the change at codon 12. All of the capsule-positive isolates detected by this method contained an arginine at position-12, suggesting that this residue may indeed be important. Mutation of Arg-12 to Gly resulted in an inactive enzyme, as determined by assays of crude lysates and complementation of EV-5. In order to confirm this result with pure enzyme, lysates of the R12G mutant strain were subject to the Yellow-3 dye-binding chromatography previously described for purification of *E. coli* CMP-NeuNAc synthetase [17]. The R12G mutant, unlike wild-type enzyme, did not bind to Yellow-3-agarose. The lack of binding to Yellow-3-agarose could be due to either a modification of the nucleotide-binding site or misfolding of the mutant enzyme. This type of change in dye-binding chromatography was observed for tryptophan binding-site mutants of the anthranilate synthase complex from *Salmonella typhimurium* [22].

Ion-exchange and gel-filtration chromatography do not rely on specific interactions with enzyme active sites and have been used in the purification of *E. coli* CMP-NeuNAc synthetase [17]. The wild-type *E. coli* CMP-NeuNAc synthetase was partially purified by ion-exchange chromatography on DEAE-Sephacel and FPLC gel filtration on Superose-12, with a 25–50% recovery of enzymic activity. Therefore the R12G mutant CMP-NeuNAc synthetase was purified by ion-exchange chromatography and FPLC gel filtration on Superose-12. Since this enzyme lacked detectable activity in crude fractions it was monitored during the purification process by affinity purified anti-(CMP-sialic acid synthetase) antibody. A highly enriched preparation of R12G, 60% purity as judged by SDS/PAGE and Western blotting, was completely inactive. Although a modification of this procedure could potentially yield pure mutant enzymes with minimal structural alteration, it is not convenient for the analysis of mutant enzymes with little or no activity. In spite of these problems we can conclude that R12 is probably a catalytically important residue.

Expression and purification of the mutant epitope-tagged CMP-NeuNAc synthetases

Nickel-immobilized metal affinity chromatography was used to purify recombinant CMP-NeuNAc synthetases with active-site mutations in a substrate- and activity-independent manner. To accomplish this, the CMP-NeuNAc synthetase gene (*neuA*) was cloned into an expression vector that appends a C-terminal sequence encoding the 11 amino acid long herpes simplex virus epitope followed by a hexahistidine tag (HSV-HIS).

Site-directed mutagenesis was performed on the HSV-HIS tag plasmid to create the full-length mutant enzymes R12A, R12K, K16A, K21A and K21R to investigate further the role of basic residues in the N-terminal motif. These tagged enzymes were

Table 1 Specific activity of purified CMP-NeuNAc synthetase mutants

N.D., no detectable activity.

NeuA-HSV-HIS mutants	Specific activity (nmol · min ⁻¹ · mg ⁻¹)
NeuA-HSV-HIS	2900
R12A	1.4
R12K	18
K16A	860
K21A	N.D.
K21R	2.8

routinely purified by DEAE-Sephacel and immobilized metal affinity chromatography to greater than 95 % purity, with yields ranging from 0.4–1.8 mg/l.

Activity of the tagged CMP-NeuNAc synthetase

The enzymic activity of the purified tagged and untagged CMP-NeuNAc synthetase was determined to ensure that the tag did not adversely affect the enzyme. The specific activity of the NeuA-HSV-HIS enzyme was similar to that of the untagged synthetase (results not shown). This is consistent with the observation reported previously [23]. *E. coli* CMP-NeuNAc synthetase containing an epitope tag at the C-terminus [23] had equivalent enzyme activity to unmodified enzyme.

The point-mutant enzymes R12A, R12K, K16A, K21A and K21R were purified and assayed to identify which of these residues are required for activity (Table 1). Only two basic residues in the N-terminal motif, R12 and K21, resulted in loss of activity after mutation. It appears that residue K16 is not essential for activity, since the mutant K16A retained approx. 30–60 % of the specific activity seen in the wild-type enzyme. The R12A substitution decreases the enzymic activity near the limits of detection of the assay, confirming the suggestion above that inactivation of the untagged enzyme by mutagenesis of R12 to glycine implies that this residue is essential. Since alanine and glycine are not conservative substitutions for arginine, the R12K mutant was tested. The enzymic activity of the R12K mutant was easily detected, demonstrating that a basic charge is required at this site. However, the enzyme possessed activity that was 100-fold below that of the wild-type enzyme, which suggests that effects other than charge may be responsible for these differences. The enzyme appears to require a lysine residue at position 21. The substitution of a positive charge at residue 21 had less of an effect than that seen by substitution of lysine for arginine at position 12. The K21A mutant had no detectable activity, whereas the activity of the K21R mutant was near the limits of detection when high concentrations of enzyme were added to the assay mixture. Lysine has a lower pK_a and a smaller radius of gyration than arginine. These residues are apparently not interchangeable.

Thermal stability of the K21 mutant synthetases

The thermal stability of the K21A and K21R mutant enzymes was determined to ascertain whether these mutations adversely affect the structure of the enzyme. Protein aggregation was used as an endpoint in these experiments, since the native enzyme was shown to become inactive and to aggregate at relatively low temperatures or under mild denaturation conditions [17]. The $T_{1/2}$ is the temperature at which one-half of the protein has

Table 2 Steady-state kinetic analysis of CMP-NeuNAc synthetase mutants

Kinetic parameters	NeuA (untagged)	NeuA-HSV-HIS	R12K-NeuA-SV-HIS	K16A-NeuA-HSV-HIS
K_m (CTP) (mM)	0.7	0.4	1.7	1.6
K_s (mM)	0.7	0.4	3.9	1.6
K_m (NeuNAc) (mM)	31	16	35	32
k_{cat} (s ⁻¹)	20	14	0.16	5.7
k_{cat}/K_m (CTP) (mM ⁻¹ · s ⁻¹)	29	36	0.063	3.6
k_{cat}/K_m (NeuNAc) (mM ⁻¹ · s ⁻¹)	0.66	0.88	0.0031	0.18

aggregated, as determined by an increase in absorption at 300 nm. The $T_{1/2}$ value for wild-type enzyme was determined to be 50.2 °C, whereas those for R12A and K21R are 51.1 °C and 49.8 °C respectively. This result shows that the K21A and the K21R mutations do not affect the thermal stability of these enzymes.

Steady-state kinetic analysis

Steady-state kinetic analysis was performed to determine what effects any of the mutations have on catalysis. Previous reports suggest that the enzyme catalyses the formation of CMP-NeuNAc via a sequential ordered mechanism [13,23]. This mechanism was assumed in the calculation of kinetic constants. The kinetic parameters demonstrate that the tag does not interfere with catalysis or substrate binding, since all of the parameters are within the same order of magnitude (Table 2). The R12K mutation resulted in a 100-fold decrease in k_{cat} , suggesting that R12 plays a role in the formation of product from the Michaelis complex in the catalytic reaction. There was a 4-fold increase in the K_m for CTP and a 10-fold increase in the kinetically determined substrate dissociation constant, K_s , suggesting that R12 may play a role in binding the substrate CTP. It is conceivable that the binding of CTP and formation of CMP-NeuNAc could both involve the interaction of substrate with R12 in a related step. The K_m for NeuNAc was essentially unchanged. Thus this amino acid does not appear to be involved in NeuNAc binding. The change in the K_s for CTP could account for the altered behaviour of the R12G mutant enzyme in dye-binding chromatography. The kinetic effects of the R12A and R12G mutations were not determined, since their very low specific activity required such large amounts of enzyme as to make the experiment impractical. The K16A mutation resulted in moderate increases in all of the kinetic parameters.

Loss of enzymic activity due to mutations at amino acid K21 suggests that this residue also plays an essential role in catalysis. Insight into the role that K21 plays in CMP-NeuNAc synthetase could not be determined by steady-state kinetic analysis due to the low activity of the K21A and K21R mutant enzymes. However, this amino acid appears to interact with CTP, based on the finding that the affinity label oCTP predominantly labels the homologous residue in the *H. ducreyi* CMP-NeuNAc [24]. Lys-19 in *H. ducreyi* CMP-NeuNAc synthetase is homologous to Lys-21 in the *E. coli* enzyme [5]. In addition to this chemical modification result, the crystal structure of the *E. coli* kpsU CMP-KDO synthetase has been determined in the presence of an iridium hexachloride anion, which is used to identify amino acids involved in phosphate binding [25]. This anion forms complexes with residues R10 and K19 in the CMP-KDO synthetase structure. These residues are on opposite sides of a

loop in the putative CMP-KDO synthetase active site and correspond to R12 and K21 in the *E. coli* K1 CMP-NeuNAc synthetase. It is possible, given this association, that both residues may interact with the phosphates of CTP. The reaction mechanisms of *E. coli* CMP-KDO synthetase and CMP-NeuNAc synthetase appear to be similar [13,14]. Efforts are underway to prepare crystalline *E. coli* CMP-NeuNAc synthetase and determine the three-dimensional structure of this enzyme. Once such a structure is determined the enzyme will be co-crystallized with sugar or nucleotide to determine the interaction of the enzyme with substrates.

D.M.S. was supported by a resident research associate fellowship from the National Research Council U.S.A. All oligonucleotides were synthesized by the Facility for Biotechnology Resources, Center for Biologics Evaluation and Research, Food and Drug Administration. We wish to thank Eric Vimr for strain EV5. We are indebted to Scott Stibitz, Margaret McGowen and Justine Vionnet for their helpful comments and suggestions in the preparation of this manuscript.

REFERENCES

- Rosenberg, A. (ed.) (1995) *Biology of the Sialic Acids*, Plenum Press, New York
- Roth, J., Rutishauser, U. and Troy, F. A. (eds.) (1993) *Polysialic Acid: from Microbes to Man*, Birkhauser Verlag, Basel
- Kean, E. L. and Roseman, S. (1966) *J. Biol. Chem.* **241**, 5643–5650
- Vann, W. F., Silver, R., Abeijon, C., Chang, K., Aaronson, W., Sutton, A., Finn, C., Lindner, W. and Kotsatos, M. (1987) *J. Biol. Chem.* **262**, 17556–17562
- Tullius, M. V., Munson, Jr., R. S., Wang, J. and Gibson, B. W. (1996) *J. Biol. Chem.* **271**, 15373–15380
- Schmelter, T., Ivanov, S., Wember, M., Stangier, P., Thiem, J. and Schauer, R. (1993) *Biol. Chem. Hoppe-Seyler* **374**, 337–342
- Rodriguez-Aparicio, L. B., Luengo, J. M., Gonzalez-Clemente, C. and Reglero, A. (1992) *J. Biol. Chem.* **267**, 9257–9267
- Vionnet, J., Concepcion, N., Warner, T. G., Zapata, G., Hanover, J. and Vann, W. F. (1999) *Glycobiology* **9**, 481–487
- Munster, A., Eckhardt, M., Potvin, B., Muhlenhoff, M., Stanley, P. and Gerardy-Schahn, R. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9140–9145
- Warren, L. and Blacklow, R. S. (1962) *J. Biol. Chem.* **237**, 3527–3534
- Guerry, P., Doig, P., Alm, R. A., Burr, D. H., Kinsella, N. and Trust, T. J. (1996) *Mol. Microbiol.* **19**, 369–378
- Haft, R. F., Wessels, M. R., Mebane, M. F., Conaty, N. and Rubens, C. E. (1996) *Mol. Microbiol.* **19**, 555–563
- Ambrose, M. G., Freese, S. J., Reinhold, M. S., Warner, T. G. and Vann, W. F. (1992) *Biochemistry* **31**, 775–780
- Kohlbrenner, W. E., Nuss, M. M. and Fesik, S. W. (1987) *J. Biol. Chem.* **262**, 4534–4537
- Zapata, G., Vann, W. F., Aaronson, W., Lewis, M. S. and Moos, M. (1989) *J. Biol. Chem.* **264**, 14769–14774
- Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (eds.) (1990) *PCR Protocols: A Guide to Methods and Applications*, pp. 177–183, Academic Press, Inc., San Diego
- Zapata, G., Roller, P. P., Crowley, J. and Vann, W. F. (1993) *Biochem. J.* **295**, 485–491
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680
- Ganguli, S., Zapata, G., Wallis, T., Reid, C., Boulnois, G., Vann, W. F. and Roberts, I. S. (1994) *J. Bacteriol.* **176**, 4583–4589
- Eyzaguirre, J. (ed.) (1987) *Chemical Modification of Enzymes: Active Site Studies*, Ellis Horwood, Chichester
- Saraste, M., Sibbald, P. R. and Wittinghofer, A. (1990) *Trends Biochem. Sci.* **15**, 430–434
- Caligiuri, M. G. and Bauerle, R. (1991) *J. Biol. Chem.* **266**, 8328–8335
- Liu, J. L.-C., Shen, G.-J., Ichikawa, Y., Rutan, J. F., Zapata, G., Vann, W. F. and Wong, C.-H. (1992) *J. Am. Chem. Soc.* **114**, 3901–3910
- Tullius, M. V., Vann, W. F. and Gibson, B. W. (1999) *Protein Sci.* **8**, 666–675
- Jelakovic, S., Jann, K. and Schulz, G. E. (1996) *FEBS Lett.* **391**, 157–161

Received 10 February 1999/28 June 1999; accepted 30 July 1999