The role of cysteine residues 129 and 329 in *Escherichia coli* K1 CMP-NeuAc synthase

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N-Acetylneuraminic acid cytidyltransferase (CMP-NeuAc synthase) of *Escherichia coli* K1 is sensitive to mercurials and has cysteine residues only at positions 129 and 329. The role of these residues in the catalytic activity and structure of the protein has been investigated by site-directed mutagenesis and chemical modification. The enzyme is inactivated by the thiol-specific reagent dithiodipyridine. Inactivation by this reagent is decreased in the presence of the nucleotide substrate CTP, suggesting that a thiol residue is at or near the active site. Site-directed mutagenesis of either residue Cys-129 to serine or Cys-329 to selected amino acids has minor effects on the specific activity of the enzyme, suggesting that cysteine is not essential for catalysis and that a disulphide bond is not an essential structural component. The limited reactivity of the enzyme to other thiol-blocking

reagents suggests that its cysteine residues are partially exposed. The accessibility and role of the cysteine residues in enzyme structure were investigated by fluorescence, c.d. and denaturation studies of wild-type and mutant enzymes. The mutation of Cys-129 to serine makes the enzyme more sensitive to heat and chemical denaturation, but does not cause gross changes in the protein structure as judged by the c.d. spectrum. The mutant containing Ser-129 instead of Cys-129 has a complex denaturation pathway similar to that of wild-type *E. coli* K1 CMP-NeuAc synthase consisting of several partially denatured states. Cys-329 reacts more readily with *N*-[¹⁴C]ethylmaleimide when the enzyme is in a heat-induced relaxed state. Cys-129 is less reactive and is probably a buried residue.

INTRODUCTION

N-Acetylneuraminic acid cytidyltransferase (EC 2.7.7.43), the enzyme that catalyses formation of CMP-NeuAc, has been isolated from both mammalian (Kean and Roseman, 1966; Schauer et al., 1980; Perez et al., 1982; Rodriguez-Aparicio et al., 1992) and bacterial sources (Blacklow and Warren, 1962; Vann et al., 1987). The enzyme is specific for the β -anomer of *N*-acetylneuraminic acid and catalyses the transfer of the anomeric oxygen to the α -phosphate of CTP (Ambrose et al., 1992).

This enzyme forms an important intermediate in the *in vivo* biosynthesis of glycoconjugates. The availability and specificity of purified sugar-activating enzymes and glycosyltransferases make these enzymes convenient tools for the synthesis of biologically relevant oligosaccharides. These synthetic sialylated oligosaccharides can aid the study of a variety of recognition processes such as neural cell adhesion molecules (Hoffman et al., 1982; Roth et al., 1988), host–cell interaction of mammalian pathogens (Suzuki et al., 1983, 1984, 1986, 1987; Loomes et al., 1984) and binding of bacterial toxins to eukaryotic glycoproteins and glycolipids (Witvliet et al., 1989; Brennan et al., 1988). The gene encoding the *Escherichia coli* enzyme is part of a gene cluster for the expression of an extracellular α (2–8) homopolymer of *N*-acetylneuraminic acid (Silver et al., 1981; Escharti et al., 1983). The *E. coli* CMP-NeuAc synthase gene (*neuA*) has been isolated (Vann et al., 1987), and the primary structure of the enzyme has been deduced from the sequence of the gene that

encodes the protein (Zapata et al., 1989). The enzyme consists of 418 amino acids, has a molecular mass of 48 621 Da and contains only two cysteine residues located at positions 129 and 329. Very little information has been published on the nature of the active-site residues of CMP-NeuAc synthase or the structure and stability of the protein. In this study, the role of the cysteines in the structural and catalytic properties of the enzyme has been examined by site-directed mutagenesis and chemical modification.

MATERIALS AND METHODS

Cells and plasmids

DH5 α -competent cells were obtained from GIBCO-BRL Life Technologies Inc. *E. coli* strain EV5 has a mutation in the CMP-NeuAc synthase gene (*neuA*) and was obtained from Dr. Eric Vimr (Department of Veterinary Pathobiology, University of Illinois, Champaign-Urbana, IL, U.S.A.). Plasmid pTTQ8 was purchased from Amersham.

Site-directed mutagenesis

The role of Cys-129 and -329 in enzyme structure and activity was investigated using site-directed mutagenesis. The PCR mutagenesis method of Nelson and Long (1989) was used to generate random mutations at position 129 or 329 in CMP-NeuAc synthase. Oligonucleotides were synthesized using an Applied Biosystems model 380B DNA synthesizer. The conditions described in the Gene Amp DNA Amplification Reagent Kit (Perkin–Elmer Cetus) were used to amplify all DNA fragments.

Abbreviations used: IPTG, isopropyl β -D-thiogalactoside; DTT, dithiothreitol; DTDP, 4,4-dithiodipyridine; NEM, *N*-ethylmaleimide; MMTS, methyl methanethiosulphonate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); BCA, BCA protein assay reagent; WT, wild type.

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Oligonucleotide A (5'-GCGAGAATTCAAAAATCAGGGG-GAATAATGAGAACAAAAATTATTGCGA-3'), contains an *EcoRI* restriction endonuclease site, a Shine-Dalgarno sequence and the ATG codon of the *neuA* gene. Oligonucleotide B (5'-GGGGTACTAGTAACCCGGGCAAGCTTTCATTTAACA-ATCTCCGCTATTT-3') contains the CMP-NeuAc synthase stop codon and a *HindIII* restriction endonuclease sequence in its 5' terminus. The underlined sequences correspond to *neuA* nucleotide sequences (Zapata et al., 1989). All molecular cloning procedures were as described by Sambrook et al. (1989).

Two additional primers were synthesized containing randomly mutated Cys-129 or Cys-329 codons, primer G (5'-AGAAA-AATACCAAXXXGTTGTTTCTGTAC-3') and primer H (5'-TATTTGAAGAAAACAXXXCAGTATATAAAG-3'). A 915 bp fragment was amplified using primers G and B, containing random mutations in the Cys-129 codon, and a 314 bp fragment using primers H and B containing random mutations in the Cys-329 codon. The fragments were purified in low-melting-point agarose gels, and 3 µl of the purified bands were mixed individually with 100 fmol of wild-type (WT) CMP-NeuAc synthase DNA. The mutated DNA fragments were extended for one cycle in the thermal cycler. Primers A and B were added to the amplification mixture and then subjected to 20 cycles. The resulting *neuA* genes containing all possible combinations of codons were ligated into the cloning vector pTTQ8.

Screening of mutant CMP-NeuAc synthases

DNA of randomly generated Cys-129 or Cys-329 mutations was transformed into DH5α cells. Cells were plated on Luria broth containing 200 µg/ml ampicillin. Transformants were pooled from the plates in 3 ml of Luria broth, diluted to 10 ml and grown for 1 h. The DNA was isolated using Quiagen mini preparation columns (Quiagen Inc., Chatsworth, CA, U.S.A.). Purified DNA containing randomly mutated *neuA* genes was used to transform *E. coli* *neuA*⁻ EV5 cells. Mutants were selected for their ability to complement EV5 on anti-K1 polysaccharide plates prepared in Davis minimal medium (Robbins et al., 1974) containing 200 µg/ml ampicillin. Complementation was detected by the formation of haloes around colonies. Transformed cells were plated on antiserum and ampicillin-containing plates as described above. Clones able to restore CMP-NeuAc synthase activity were isolated and their DNA was extracted. The mutant DNA was retransformed into DH5α cells and the transformants were assayed for synthase activity using the thiobarbituric acid method (Vann et al., 1987).

Expression and purification of CMP-NeuAc synthases

CMP-NeuAc synthase was purified using affinity chromatography as described by Vann et al. (1987), or using Reactive Yellow 3-agarose (Sigma) as described by Shames et al. (1991). Luria broth (1 litre) containing 200 µg of ampicillin was inoculated with *E. coli* DH5α cells containing the desired mutant plasmid. The cells were grown until $A_{600} = 1.0$ and induced for 3 h with 1 mM isopropyl β-D-thiogalactoside (IPTG). The cells were harvested by centrifugation, and lysed using a French press. The soluble portion of the cell lysate was fractionated using (NH₄)₂SO₄. The 35–60%-satd. (NH₄)₂SO₄ fraction containing CMP-NeuAc synthase was dialysed against 0.05 M Bicine/1 mM dithiothreitol (DTT), pH 7.6, and the protein solution applied to a 20 ml Reactive Yellow 3-agarose column. Unbound proteins were removed by washing with 0.05 M Bicine/1 mM

Table 1 Sequences of mutagenic primers and random mutations in the structural gene for *E. coli* K1 CMP-NeuAc synthase

Mutant	Amino acid change	Mutagenic sequence	Relative specific activity
C129C	Cys→Cys	TGC	100
C129N	Cys→Asn	AAT	64
C129S	Cys→Ser	TCG	100
C129I	Cys→Ile	ATC	16
C129Y	Cys→Tyr	TAC	1
C129F	Cys→Phe	TTT	21
C329Y	Cys→Tyr	TAT	116
C329T	Cys→Thr	ACA	64
C329H	Cys→His	CAC, CAT	111

DTT, pH 7.6, buffer. CMP-NeuAc synthase was eluted from the column using 5 mM CTP in the same buffer.

Immunoelectrophoresis

E. coli strain DH5α harbouring selected mutant *neuA* genes was grown in 20 ml of Luria broth in the presence of 200 µg/ml ampicillin and induced with 1 mM IPTG for 3 h. Cell lysates were prepared by sonication and electrophoresed on 4–20% gradient SDS/polyacrylamide gels (Integrated Separation Systems). Separated proteins were transferred to nitrocellulose paper (Bio-Rad) and CMP-NeuAc synthase was detected with affinity-purified rabbit anti-(CMP-NeuAc synthase) antibody (Vann et al., 1987) at 1/1000 dilution as described (Towbin et al., 1979; Burnette, 1981). The enzyme was detected with alkaline phosphatase conjugated to goat anti-rabbit antibody (Promega). Rabbit anti-(CMP-NeuAc synthase) antibody (Vann et al., 1987) was purified by adsorption of the globulin fraction to a CMP-NeuAc synthase-agarose column coupled by CNBr activation. The specifically adsorbed antibody was eluted with 3 M MgCl₂.

Nucleotide sequencing of mutant *NeuA* genes

DNA was extracted from *E. coli* harbouring mutant plasmids using the Quiagen midi prep system (Quiagen). Nucleotide sequencing was performed by the procedure of Sanger et al. (1977), using Sequenase (United States Biochemical Corp.). Synthetic oligonucleotide primers, located upstream from the cysteine codons and complementary to the sequence of the *neuA* gene, were used in sequencing reactions according to the manufacturer's protocol. The polyacrylamide/urea gel electrophoresis system described by Biggen et al. (1983) was used for the separation of the DNA fragments.

Protein determination

The concentration of purified CMP-NeuAc synthase was calculated using either the molar absorption coefficient at 278 nm, the Bradford method (Bradford, 1976) or BCA protein assay (Lane et al., 1986; Nugent et al., 1986). The calculated molar absorption coefficient at 278 nm was determined to be 54.8 mM⁻¹·cm⁻¹ based on the amino acid composition (Gill et al., 1989). The Bradford method was used to determine protein concentration of crude lysates, and BCA was used to calculate protein concentration after labelling with *N*-[¹⁴C]ethylmaleimide ([¹⁴C]NEM). BSA was used as the standard.

Figure 1 Thiol-blocking reagents used with CMP-NeuAc synthase

Enzyme activity

Enzyme activity was measured by the thiobarbituric acid method as described (Vann et al., 1987).

Spectrophotometric determination of thiol groups

Purified WT CMP-NeuAc synthase and the mutant enzymes C129S and C329T (1–2 mg/ml) were dialysed against 50 mM Bicine, pH 8. The protein (250 μ l) was mixed with 250 μ l of 2 mM 4,4'-dithiodipyridine (DTDP) in 0.05 M Bicine, pH 8, and the absorbance at 324 nm was recorded with time. The DTDP solution was calibrated before each determination using cysteine in 0.05 M Bicine, pH 8, as a reference standard.

Chemical modification with thiol-blocking reagents

A solution of CMP-NeuAc synthase (50–100 μ g/ml) in 50 mM Bicine/0.3 M NaCl, pH 7.6, was adjusted to 1 mM thiol-blocking reagent [NEM, DTDP, methyl methanethiosulphonate (MMTS) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)] (Figure 1). Portions of 20 μ l were taken periodically, diluted in 80 μ l of distilled water and mixed with 150 μ l of an enzyme assay cocktail containing 0.3 M Bicine, 32 mM $MgCl_2$, 8.8 mM CTP and 4.5 mM NeuAc, pH 9. The mixture was incubated at 37 °C for 30 min and the CMP-NeuAc formed measured colorimetrically (Vann et al., 1987). Temperature-dependent inactivation experiments were performed following the same procedures with WT CMP-NeuAc synthase and the mutant enzyme C329T at 42 °C and mutant C129S at 40 °C.

Labelling of enzyme with [^{14}C]NEM and CNBr fragmentation

CMP-NeuAc synthase (450 μ g; 9.3 nmol) was incubated with 100 nmol [^{14}C]NEM (38.7 mCi/nmol) in 468 μ l of 0.05 M Bicine, pH 8, at the desired temperature. Samples (100 μ l) were removed periodically, quenched with 10 mM cysteine, and the protein was precipitated with 10% trichloroacetic acid. After 45 min at room temperature, the precipitate was washed with 10% trichloroacetic acid and redissolved in 100 μ l of 0.1 M NaOH for radioactive counting and protein determination by BCA.

[^{14}C]NEM-labelled CMP-NeuAc synthase was cleaved with CNBr at the end of the incubation period by mixing 30 μ l of labelled enzyme with a 70 μ l solution of CNBr (250 mg/ml) in 90% formic acid. After 4 h at 37 °C, 1 ml of water was added

and the reagents were removed by repeated evaporation *in vacuo* with water. The peptides were resuspended in SDS/PAGE sample buffer and electrophoresed on 16% polyacrylamide gels (Laemmli, 1970). Radioactive peptides were detected by autoradiography.

C.d. and fluorescence spectroscopy

C.d. spectra were recorded at room temperature in the wavelength range 195–260 nm, on a JASCO model J-500A/DP501N spectropolarimeter, in Hellma QH cells with a path-length of 1 mm. Measurement of the mean residue ellipticity at 222 nm, $[\theta]_{222}$, provided an upper-limit estimate of the fractional α -helix content of the protein using the assumption, that for 100% helix, $\theta_{222} = 39\,500$ degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$ (1-2.6/418) (Chen et al., 1974).

Fluorescence spectra were recorded on a Perkin-Elmer model LS-5B luminescence spectrometer. The excitation maximum was empirically determined to be 295 nm and used to record emission spectra. Enzyme solutions used in denaturation experiments were prepared by diluting a concentrated stock solution 10-fold to the desired guanidinium chloride, urea or SDS concentration. Fluorescence and c.d. were recorded on enzyme solutions buffered in 20 mM Tris/20 μ M DTT, pH 7.5.

RESULTS

E. coli CMP-NeuAc synthase is inhibited by the substrate analogue 5-mercurial-CTP (Vann et al., 1987) but the enzyme does not require the presence of reducing agents for enzyme activity. Incubation with 5 mM *p*-chloromercuribenzoate causes a 30% loss of activity, and 1 mM mercuric chloride causes a loss of 80% activity after a 1 h incubation. These results suggest a possible role of thiol groups in enzyme function. Thus the role and accessibility of Cys-129 and Cys-329 were investigated.

Surface accessibility of thiol groups

The disulphide reagent DTDP rapidly inactivates the enzyme at 1 mM with $t_{1/2} = 22$ min (Figure 2). The enzyme is protected from inactivation by the nucleotide substrate CTP. Thiol-

Figure 2 Inactivation of WT CMP-NeuAc synthases by DTDP at room temperature

WT CMP-NeuAc synthase (50–100 μ g/ml) in 50 mM Bicine/0.3 M NaCl, pH 7.6, was incubated at room temperature with 1 mM DTDP, O, WT alone; ●, WT + 1 mM DTDP; ■, WT + 1 mM DTDP + 16 mM CTP.

Figure 3 DTDP titration of thiol groups of WT CMP-NeuAc synthase in the presence and absence of 2.5% SDS

CMP-NeuAc synthase (1–2 mg/ml) in 250 μ l of 0.05 M Bicine, pH 8, was mixed with 250 μ l of DTDP solution at a concentration of 2 mM in 0.05 M Bicine, pH 8.0. Absorbance was recorded with times at 324 nm in the presence of 2.5% SDS (○) or the absence of SDS (●). The concentration of enzyme was estimated spectrophotometrically before each determination, assuming the absorption coefficient based on the amino acid composition.

blocking reagents having a range of size and polarity were tested for their reactivity toward CMP-NeuAc synthase (Figure 1). The disulphide reagents DTNB and MMTS react very slowly with CMP-NeuAc synthase. DTNB reaction resulted in only a 15–30% loss in enzymic activity after 1 h. However, 70% inactivation was observed with the smallest reagent, MMTS, after 3 h. CMP-NeuAc synthase is not inactivated by the alkylating reagents iodoacetic acid, iodoacetamide or NEM at room temperature. Attempts to label the enzyme with iodo[14 C]acetic acid were unsuccessful.

Inactivation of CMP-NeuAc synthase with DTDP is accompanied by a corresponding reaction of 1 mol equiv. of thiol per 48 621 Da as measured at A_{324} upon complete inactivation by DTDP (Figure 3). In a second experiment to determine the availability of thiol groups, SDS was added as a denaturant. The number of residues titratable with DTDP was not increased by the addition of 2.5% SDS to the reaction mixture. Reaction of thiol-containing residues with DTNB could not be detected spectrophotometrically during a 1 h incubation. Thus the thiol-

containing residues of *E. coli* CMP-NeuAc synthase are not completely exposed to solvent but react most rapidly with the uncharged reagent DTDP. Enzyme is protected against inactivation in the presence of the substrate, 10 mM CTP (Figure 2). Thus there appears to be one cysteine residue at or near the active site.

Site-directed mutagenesis of residues Cys-129 and Cys-329

The cysteine residues of CMP-NeuAc synthase were mutated by random mutagenesis. The relative specific activities of the mutant enzymes and amino acid substitutions are given in Table 1. Both cysteine residues can be substituted without complete loss of activity. This indicates that neither of these residues is essential for catalysis even though their modification or substitution may affect enzyme activity. Furthermore, as each residue can be individually substituted, a disulphide bond is not essential for the formation of an active enzyme structure. Cys-329 can be changed to a histidine or tyrosine without a decrease in activity, or to a threonine with less than 50% loss in specific activity. Tyrosine and histidine have larger radii of gyration and different polarity characteristics from cysteine (Bordo and Argos, 1991), indicating that the enzyme can accommodate large changes in side chains at residue 329 without loss of enzyme activity. As might be expected, enzymes generated by this random mutagenesis procedure in which cysteine was conserved were equally as active as the parent enzyme. On the other hand large changes in side chain structure are not readily accommodated at residue 129. Of the substitutions isolated by this technique, only mutant enzyme C129S having a serine at residue 129 has a specific activity as high as the WT enzyme. The least active mutant enzyme has a tyrosine at this position.

Thermal stability

As these thiol-containing residues in *E. coli* CMP-NeuAc synthase are not essential for catalytic activity, their role in the structure of CMP-NeuAc synthase was investigated. The thermal stability of the WT enzyme and mutant enzyme activities were compared. WT CMP-NeuAc synthase loses activity slowly (half-life greater than 90 min) at 42 °C but loses activity rapidly at 46 °C (Figure 4a). Loss of activity at 46 °C is accompanied by aggregation and turbidity of enzyme solutions. At 42 °C the

Figure 4 Thermal stability of WT CMP-NeuAc synthase and mutant C129S at different temperatures

(a) WT incubated at 40 °C (●), 42 °C (○), 46 °C (■) before assay at 37 °C; (b) WT incubated at 42 °C (○) and mutant C129S at 42 °C (●) before standard assay.

Figure 5 C.d. spectra of native and mutant CMP-NeuAc synthase

Spectra were taken at 25 °C in 0.05 M Bicine, pH 9. Protein concentration was 5 μ M. —, WT CMP-NeuAc synthase; ---, mutant C129S CMP-NeuAc synthase in guanidinium chloride.

mutant enzyme C129S rapidly loses activity (Figure 4b), suggesting that Cys-129 may be involved in maintaining structural stability in the WT enzyme. Although substitution of threonine at residue 329 results in an enzyme that is less stable at 42 °C than WT (results not shown), this enzyme nevertheless is more stable than mutant enzyme C129S.

Denaturation of CMP-NeuAc synthase

The effect of Cys-129 to serine substitution on structure was investigated by fluorescence spectroscopy and c.d. of the WT and mutant enzymes. CMP-NeuAc synthase contains three tryptophan residues. The fluorescence spectra of the mutant C129S and WT enzyme do not differ. The emission maxima for the tryptophan fluorescence is at 340 nm, suggesting that the tryptophan residues are buried in the native state of both proteins and not readily accessible to solvent. Similarly, the c.d. spectrum of the C129S does not differ from that of the WT enzyme within the wavelength range 190–240 nm. The enzymes have negative ellipticity maxima at 209 and 220 nm characteristic of an α -helix (Figure 5). The estimated helical content is 36%. These experiments indicate that mutation of Cys-129 to serine does not result in gross changes in the protein structure. Perhaps a more sensitive indication of structural change is sensitivity to denaturants. An analysis of the denaturation of these enzymes by guanidinium chloride and urea as measured by activity, fluorescence and c.d. revealed two findings: (a) the denaturation pathway of CMP-NeuAc synthase is complex and (b) the mutant C129S is more easily denatured than the WT protein. Activity was measured as a function of denaturant. WT enzyme was inactive at 1.5 M guanidinium chloride or 4 M urea whereas C129S was inactive at 1 M guanidinium chloride or 3 M urea, although both proteins appear to retain structure as judged by the c.d. (results not

shown) and fluorescence spectra. The complete denaturation of both WT and mutant C129S enzymes to a random coil structure occurs only at 5.5 M guanidinium chloride. These results indicate that the catalytic activity of CMP-NeuAc synthase is lost due to subtle changes in protein structure at medium temperatures or mild denaturants such as 1.5 M guanidinium chloride or 2.5% SDS. The cysteine residue at position 129 contributes to the stability of the active state.

Reactivity of Cys-329 and Cys-129

In the experiments described above the cysteines appear to have limited access to modifying reagents. Only one residue reacted with DTDP resulting in modification of a residue which may be near the active site. The location of this residue was investigated by chemical modification of the mutant enzymes. The WT enzyme (Figure 2) and mutant C129S (Figure 6) are inactivated by DTDP, although the mutant enzyme is inactivated at a lower rate. CTP also protects mutant C129S against inactivation (Figure 6). Inactivation of both WT and C129S mutant enzyme parallels the modification of a single thiol group. Both WT and C129S can be re-activated by DTT. Furthermore both WT and C129S enzymes are inactivated by NEM, DTNB and MMTS at 42 and 40 °C respectively, temperatures slightly lower than that required for thermal inactivation. Both enzymes are inactivated at a lower rate with 1 mM DTNB and NEM in the presence of 10 mM CTP. A single thiol residue is detected spectrophotometrically with DTNB under these conditions. DTDP also inactivates the mutant enzyme C329S with a 1:1 stoichiometry. The incorporation of DTDP into this mutant enzyme is slower than in C129S and WT enzymes and is also reversible by DTT, releasing 1 mol of thiopyridone. The reaction of Cys-129 and Cys-329 with DTDP may be mutually exclusive in the WT enzyme. This conclusion follows from the observation that only one of the two available residues can be titrated in WT and both mutant enzymes react with DTDP with a 1:1 stoichiometry.

The relative reactivity of Cys-129 and Cys-329 in the WT enzyme was further investigated by labelling with [14 C]NEM. WT and mutant enzymes C129S and C329T were tested with labelled NEM at room temperature and the higher temperature (Table 2).

Figure 6 Inactivation of mutant C129S CMP-NeuAc synthase by DTDP at room temperature

CMP-NeuAc synthase C129S mutant (50–100 μ g/ml) was inactivated by 1 mM DTDP in 50 mM Bicine/0.3 M NaCl, pH 7.6, and incubated at room temperature. ■, C129S alone; ●, C129S + 1 mM DTDP; ○, C129S + 1 mM DTDP + 10 mM CTP.

Table 2 Quantitative labelling of synthase proteins using [^{14}C]NEM

For the WT enzyme, the two results given were obtained at room temperature and 42 °C respectively, and, for the mutant enzymes, the two results were obtained at room temperature and 40 °C respectively. ND, not determined.

Time (min)	Labelling (nmol of NEM/nmol of protein)			
	WT	WT + DTDP	C129S	C329T
0	0	0	0	0
15	0.065/1.14	ND	0.22/0.79	ND
30	0.10/1.28	ND	0.24/0.94	ND
45	0.13/1.34	0.09/0.07	0.29/0.98	0.05/0.34

At 42 °C 1.34 equiv. of NEM was incorporated into the WT and 0.13 equiv. at room temperature. Similarly, 0.98 equiv. of [^{14}C]NEM was incorporated into C129S at 40 °C and 0.29 equiv. at room temperature. Mutant enzyme C329T incorporated the least [^{14}C]NEM at elevated temperatures, suggesting that Cys-129 is the most hindered residue. As NEM can also react with other nucleophiles in the protein, the specificity of the reaction was tested by incubation of WT enzyme with DTDP for 1 h before labelling with [^{14}C]NEM. DTDP blocked labelling of the WT enzyme. Only 0.1 mol of NEM was incorporated into the WT enzyme at 42 °C which had been previously treated with DTDP.

Modified enzymes were fragmented with CNBr and subjected to SDS/PAGE autoradiography. The molecular masses of the observed CNBr fragments were compared with those predicted for cleavage from the amino acid sequence. On the basis of the amino acid sequence, CNBr cleavage of WT CMP-NeuAc synthase should yield two labelled fragments corresponding to residues 45–200 (molecular mass 18 092 Da) and residues 213–393 (molecular mass 21 445 Da). Only one of these bands was labelled in the mutant enzymes. As these bands only appeared in digests of enzymes containing the appropriate thiol group, the predominant reactivity with [^{14}C]NEM was probably due to reactivity with Cys-129 or Cys-329.

This result suggests that residue 329 is accessible after partial denaturation and that residue 129 is buried in the interior of the protein and therefore less accessible. In an attempt to determine the reactivity of residue 129, WT enzyme was titrated with the disulphide reagent DTDP in the presence of denaturants. Mild denaturing conditions such as 2.5% SDS resulted in rapid titration of only one thiol (Figure 3) and, as indicated above, did not change the fluorescence spectrum of the protein. No thiol residues could be detected in 6 M guanidinium chloride. This could be explained by the rapid formation of a disulphide bond on complete denaturation of the WT enzyme.

DISCUSSION

E. coli CMP-NeuAc synthase contains two thiol residues of different accessibility. Site-directed mutagenesis demonstrates that these residues are not essential for catalysis despite the sensitivity of the enzyme to thiol-blocking reagents. Re-examination of the role of cysteine in several enzymes such as *Drosophila* alcohol dehydrogenase (Chen et al., 1990) by site-directed mutagenesis has demonstrated that these residues are not essential for catalysis despite chemical modification experiments suggesting that they are located in or near the active site.

Nevertheless, one cannot rule out the location of a non-essential thiol near the binding site of CTP, as this substrate protects against chemical modification by thiol-blocking reagents.

Chemical modification and denaturation studies on WT and mutants show that neither Cys-129 nor Cys-329 is readily available to solvent. One of the residues, probably Cys-329, is reactive toward NEM, DTNB and MMTS after a partial opening of the protein as a result of exposure to elevated temperatures. This change in structure does not result in a significant change in helical content or the degree of exposure of the buried tryptophan residues. The fluorescence spectrum of the enzyme is unaltered in 2.5% SDS even though a single thiol now reacts rapidly with DTNB.

Replacements at residue 129 are more sensitive to the structure of the amino acid side chain than replacements at residue 329. Side chain replacements at residue 129 with the least effect on enzyme activity have side chain volumes equal to or less than that of cysteine [approx. 50 Å³ (0.05 nm³). This observation is consistent with the apparent buried nature of this residue (Bordo and Argos, 1990). Residue 329 is probably in a partially buried region of the protein. In addition to the effect of size of residue 129 on activity, the high specific activity of mutant enzyme C129S suggests that the hydrogen of the thiol group on Cys-C129 may play a role in the formation of a fully active enzyme.

The denaturation pathway of *E. coli* CMP-NeuAc synthase is complex. It appears that the enzyme first undergoes a subtle change in structure leading to aggregation, presumably as a result of the exposure of hydrophobic residues. Enzyme in this state is no longer active and is solubilized by higher concentrations of guanidinium chloride. Complete denaturation requires high concentrations of urea or guanidinium chloride. The aggregation is also observed in the presence of urea suggesting that it is not salt-induced. Nevertheless, steps in the denaturation of *E. coli* CMP-NeuAc synthase are observable by tryptophan fluorescence, aggregation or c.d. The complex nature of the denaturation and the fact that the enzyme has only two thiols make it an interesting model for observing the role of cysteine in protein folding and stability.

REFERENCES

- Ambrose, M. G., Freese, S. J., Reinhold, M. S., Warner, T. G. and Vann, W. F. (1992) *Biochemistry* **31**, 775–780
- Biggen, M. D., Gibson, T. J. and Hong, G. F. (1983) *Proc Natl. Acad. Sci. U.S.A.* **80**, 3963–3965
- Blacklow, R. S. and Warren, L. (1962) *J. Biol. Chem.* **237**, 3520–3534
- Bordo, B. and Argos, P. (1990) *J. Mol. Biol.* **211**, 975–988
- Bordo, D. and Argos, P. (1991) *J. Mol. Biol.* **217**, 721–729
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brennan, M. J., David, J. L., Kenimer, J. G. and Manclark, C. R. (1988) *J. Biol. Chem.* **263**, 4895–4899
- Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195
- Chen, Y.-H., Yang, J. T. and Chau, K. H. (1974) *Biochemistry* **13**, 3350–3359
- Chen, Z., Lu, L., Shirley, M., Lee, W. R. and Chang, S. (1990) *Biochemistry* **29**, 1112–1118
- Escharti, C., Hirschel, B., Boulnois, B. J., Varky, J. M., Wald-voguel, F. and Timmis, K. N. (1983) *Infect. Immun.* **41**, 54–60
- Gill, S. C., Stanley, P. H. and von Hippel, P. H. (1989) *Anal. Biochem.* **182**, 319–326
- Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. A. and Edelman, G. M. (1982) *J. Biol. Chem.* **257**, 7720–7729
- Kean, E. L. and Roseman, S. (1966) *J. Biol. Chem.* **241**, 5643–5650
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lane, D., Federman, D., Flora, J. L. and Beck, B. L. (1986) *J. Immunol. Methods* **92**, 261–270
- Loomes, L. M., Uemura, K., Childs, R. A., Rogers, G. N., Scudder, P. R., Michalski, J. C., Hounsell, E. F., Taylor-Robinson, D. and Feizi, T. (1984) *Nature (London)* **307**, 560–563
- Nelson, R. M. and Long, G. L. (1989) *Anal. Biochem.* **180**, 147–151
- Nugent, D. W., Doyle, C. and Fottrell, P. F. (1986) *Clin. Chem.* **33**, 1671

- Perez, M., Vannier, A. and Hirschberg, C. B. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 662
- Robbins, J. B., McCracken, G. H., Jr., Gotschlich, E. C., Orskov, F., Orskov, I. and Hanson, L. A. (1974) *N. Engl. J. Med.* **290**, 1216–1220
- Rodriguez-Aparicio, L. B., Luengo, J. M., Gonzalez-Clemente, C. and Reglero, A. (1992) *J. Biol. Chem.* **267**, 9257–9263
- Roth, J., Zuber, C., Wagner, P., Taatjes, D. J., Weisberg, C., Heitz, P. U., Goridis, C. and Bitter-Suermann, D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2999–3003
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Schauer, R., Haverkamp, J. and Ehrlich, K. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 641–648
- Shames, S. L., Simin, E. S., Christopher, C. W., Schmid, W., Whitesides, M. and Yang, L. (1991) *Glycobiology* **1**, 187–191
- Silver, R. P., Finn, C. W., Vann, W. F., Aaronson, W., Schneerson, R., Kretschnner, P. J. and Garon, C. F. (1981) *Nature (London)* **289**, 696–698
- Suzuki, Y., Suzuki, T. and Matsumoto, M. (1983) *J. Biochem. (Tokyo)* **93**, 1621–1633
- Suzuki, T., Harada, M., Suzuki, Y. and Masumoto, M. (1984) *J. Biochem. (Tokyo)* **95**, 1193–1200
- Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K. and Nobusawa, E. (1986) *J. Biol. Chem.* **261**, 17057–17061
- Suzuki, Y., Nagao, Y., Hideshige, K., Suzuki, T., Matsumoto, M. and Murayama, J. (1987) *Biochim. Biophys. Acta* **903**, 417–424
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350
- Vann, W. F., Silver, R., Abeijon, C., Chang, K., Aaronson, W., Sutton, A., Finn, C. W., Lindner, W. and Kotsatos, M. (1987) *J. Biol. Chem.* **262**, 17556–17562
- Witvliet, M. H., Burns, D. L., Brennan, M. J., Poolman, J. T. and Manclark, C. R. (1989) *Infect. Immun.* **57**, 3324–3330
- Zapata, G., Vann, W. F., Aaronson, W., Lewis, M. S. and Moos, M. (1989) *J. Biol. Chem.* **264**, 14769–14774