# $^{13}C$ NMR Investigation of the Anomeric Specificity of CMP–*N*-Acetylneuraminic Acid Synthetase from *Escherichia coli*†

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ABSTRACT: The anomeric specificity of *Escherichia coli* CMP–*N*-acetylneuraminic acid (CMP–NeuAc) synthetase was investigated by NMR using $^{13}C$-labeled *N*-acetylneuraminic acid (NeuAc). Consumption of the β-anomer of [2-$^{13}C$]*N*-acetylneuraminic acid was observed upon addition of enzyme, with a concomitant appearance of an anomeric resonance for CMP–*N*-acetylneuraminic acid. Inhibition by substrate analogues confirms the importance of the anomeric center for interaction of substrate with the enzyme. The fate of the anomeric oxygen was determined in a similar manner using [2-$^{13}C$,(50 atom %)$^{18}O$]*N*-acetylneuraminic acid. An upfield shift of 1.5 Hz in the anomeric resonance of both the [$^{13}C$]NeuAc substrate and CMP–[$^{13}C$]NeuAc product was observed due to the $^{18}O$ substitution. This result implies conservation of the NeuAc oxygen. Results of steady-state kinetic analysis suggest a sequential-type mechanism and therefore no covalent intermediate. Thus, CMP–β-NeuAc is probably formed by a direct transfer of the anomeric oxygen of β-NeuAc to the α-phosphate of CTP.

The capsular polysaccharide of *Escherichia coli* K1 is an α(2–8) homopolymer of *N*-acetylneuraminic acid (NeuAc) (DeWitt & Rowe, 1961; McGuire & Binkley, 1964). It is the most commonly occurring capsule associated with *E. coli* disease (Robbins et al., 1974, 1980; Kaijser et al., 1977). The poly(*N*-acetylneuraminic acid) capsules of *E. coli* are formed via the transfer of NeuAc from cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) (Troy et al., 1982); this activated donor molecule is unusual because it is a sugar monophosphoryl derivative instead of the more common sugar diphosphoryl nucleoside (Gabriel, 1982). Another example of a sugar donor molecule of this form is CMP–3-deoxy-D-*manno*-octulosonate (CMP–KDO); its formation is catalyzed by the enzyme CMP–KDO synthetase (Ghalambor et al., 1963a,b, 1966; Ghalambor & Heath, 1966; Ray, 1981).

*N*-Acetylneuraminate cytidyltransferase (EC 2.7.7.43), the enzyme that catalyzes formation of CMP–NeuAc, has been isolated from both mammalian (Kean & Roseman, 1966; Schauer et al., 1980; Perez et al., 1982) and bacterial sources (Blacklow & Warren, 1962a,b; Kean & Roseman, 1966; McGuire, 1976). 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. This study examines the specificity of CMP–*N*-acetylneuraminic acid synthetase for the anomeric forms of *N*-acetylneuraminic acid and seeks to ascertain the fate of the anomeric oxygen atom. Its mechanism will also be compared with that of the closely analogous CMP–KDO synthetase, which has been investigated by Kohlbrenner et al. (1985, 1987). It was demonstrated by Kohlbrenner et al. (1985) that the CMP–KDO synthetase transfers the anomeric oxygen of the least prevalent β-KDO to the α-phosphate of CTP.

## Materials and Methods

*CMP–NeuAc Synthetase.* The isolation and purification of *N*-acetylneuraminate cytidyltransferase (CMP–NeuAc synthetase) from *E. coli* has been described previously (Vann et al., 1989).

*Reagents.* Cytidine triphosphate (CTP) and *N*-acetylneuraminic acid (NeuAc) were obtained from Sigma Chemical Co. and used without purification. CTP was dissolved in water and adjusted to pH 7 with sodium hydroxide. The solutions used for enzymatic studies were prepared by sequential 1:1 dilutions using volumetric glassware. *N*-Acetylneuraminic acid solutions were prepared analogously except for the pH adjustment.

*Preparation of [2-$^{13}C$]N-Acetylneuraminic Acid.* [2-$^{13}C$]*N*-Acetylneuraminic acid was enzymatically prepared from *N*-acetyl-D-mannosamine (Sigma Chemical Co.) and [2-$^{13}C$]sodium pyruvate (MSD Isotopes) using *N*-acetylneuraminic acid aldolase (Sigma Chemical Co.) as a catalyst (Roseman & Comb, 1960). The reaction was carried out in 0.6 mL of 0.020 M potassium phosphate buffer at pH 7.2 using 225 mg of *N*-acetyl-D-mannosamine, 94 mg of sodium [2-$^{13}C$]pyruvate, and 100 μL of NeuAc aldolase solution (1 unit). The reaction was monitored colorimetrically by assaying a 2-μL aliquot for NeuAc produced using thiobarbituric acid (Warren & Blacklow, 1962; Vann et al., 1987). After 22 h, a yield of 39% was indicated. Additional NeuAc aldolase solution (25 μL) was added, and a 51% conversion was observed after 50 h. The reaction was terminated by adding 41 mg of trichloroacetic acid. After centrifugation, the precipitate was washed with 100 μL of water containing 9 mg of trichloroacetic acid. The combined supernatant solutions were extracted with ethyl ether (4 × 1 mL), and the aqueous phase applied to a Dowex AG-1 column (acetate form, 14 cm × 2.5 cm) and eluted with a linear gradient of 0–3 M acetic acid. Fractions were collected every 15 mL, and the [2-$^{13}C$]NeuAc was localized using the thiobarbituric acid assay. The fractions

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containing [2-$^{13}$C]NeuAc were lyophilized, yielding 153 mg.

*Preparation of [2-$^{13}$C,(50 atom %)$^{18}$O/N-Acetylneuraminic Acid.* Incorporation of $^{18}$O was accomplished by allowing [2-$^{13}$C]NeuAc to mutarotate in the presence of $H_2{}^{18}O$ (Aldrich Chemical Co., 97 atom %). This reaction was carried out directly in a 5-mm NMR sample tube. [2-$^{13}$C]*N*-Acetylneuraminic acid (3.5 mg) was dissolved in 300 μL of $H_2{}^{18}O$, 25 μL of buffer solution containing 1.87 M glycine and 0.5 M $MgCl_2$ at pH 9.4, and 70 μL of $^2H_2O$ (MSD Isotopes, "100 atom %"). Incorporation of $^{18}$O was apparent after 2 h at 37 °C by the appearance of an additional resonance signal 1.5 Hz upfield from the [2-$^{13}$C]NeuAc resonance at 98.2 ppm.

*Preparation of Methyl-5-acetamido-4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-*D*-erythro-*L*-glucononate.* 2,3-Dehydro-*N*-acetylneuraminic acid (10 mg, 34 μmol) (Warner, 1987) was dried over phosphorus pentoxide in vacuo and acetylated in a 1:1 mixture of acetic anhydride/pyridine at 4 °C overnight. The reaction was terminated after 4 h by the addition of methanol and purified on a 0.5 × 3 cm Dowex 50 × 8 $H^+$ column in methanol. The fraction containing the acetylated sugar was dried under a stream of nitrogen and esterified with diazomethane [generated from 1-methyl-3-nitro-1-nitrosoguanidine (380 μmol)] in 4 mL of ether for 30 min at 4 °C. Solvents were removed, and the residue suspended in 2 mL of 2-propanol and hydrogenated at room temperature over Pd/charcoal (1 mg) at 1 psi $H_2$ pressure. After 2 h the catalyst was removed by filtration and the product purified by HPLC on a 4.6 mm × 25 cm Ultrasphere column (Beckman Instruments, Inc.) and eluted with a linear gradient of 30% acetonitrile–water to 100% acetonitrile at 3 mL/min (yield 5 mg).

$^1$H NMR spectra were recorded in $CDCl_3$: δ 1.895 ppm (s, 4 H, acetamido methyl overlap with axial H3); 2.045–2.14 ppm (3 s, 12 H); 2.41 (m, 1 H, equatorial H3); 4.046 (dd, 2 H, H2 overlap with H5). In order to verify the orientation of H2, a homonuclear decoupling experiment (Martin & Zekter, 1988) was carried out by irradiating the H3-axial signal and observing the collapse of the H2–H5 doublet of doublets at 4.046. The H2–H3 axial coupling identified in this manner gave a coupling constant of 12 Hz, which is consistent with an axial orientation of H2. Elemental analysis was determined by high-resolution FAB mass spectrometry using a JEOL HX110hf mass spectrometer. Glycerol served as a calibration standard. Calculated mass + $H^+$ for $C_{20}H_{29}O_{12}N$ = 476.1768; found = 476.1788.

*Preparation of 5-Acetamido-2,6-anhydro-3,5-dideoxy-*D*-erythro-*L*-glucononate (2-Deoxy-N-acetylneuraminic acid).* The above compound was deesterified by suspending 5 mg in 0.5 mL of methanol and 0.1 mL of 1 N NaOH for 1 h. The reaction mixture was applied to Dowex 50 × 8 $H^+$ and eluted with water. The yield was 2.5 mg.

*NMR Instrumentation.* The $^{13}$C NMR studies were done using a General Electric GN-300 NMR spectrometer operating in the Fourier transform mode at a frequency of 75.5 MHz using a $^1$H/$^{13}$C dual probe; all experiments, except where noted, were conducted at 37 °C. The following instrumental parameters were used for the experiment using [1-$^{13}$C]NeuAc: sweep width, 2342 Hz; number of data points, 4096; repetition rate, 875.4 ms; pulse width, π/6; and number of acquisitions, 1024. The following instrumental parameters were used for the experiment using [2-$^{13}$C, (50 atom %)$^{18}$O]NeuAc: sweep width, 3175 Hz; number of data points, 8192; repetition rate, 1.29 s; pulse width, π/6; and number of acquisitions, 1024. Data were zero-filled before Fourier transformation in the latter experiment. Elapsed time reported for both NMR experiments was measured from the time of addition of CMP–NeuAc synthetase to the middle of data acquisition (13 min). Deuterium oxide was used for field/frequency lock.

*Rate of CMP–NeuAc Synthetase-Catalyzed Formation of CMP-[2-$^{13}$C]N-Acetylneuraminic Acid.* The rate of consumption of [2-$^{13}$C]NeuAc to form CMP–NeuAc was followed by measuring the area of the C-2 signals in the $^{13}$C NMR spectra. The reaction mixture at pH 8.5 is described in the figure legends and was initiated by the addition of 70 μg of CMP–NeuAc synthetase [2.7 μmol/(min·mg)] to the reaction mixture. Because pH 7.1 is below the pH optimum for the enzyme in the presence of $Mg^{2+}$, the rate of the enzymatic reaction at pH 7.1 was measured under the following conditions. [2-$^{13}$C]NeuAc (3.2 mg, 10.4 μmol) was dissolved in a solution composed of 100 μL of buffer (1.0 M bicine, 20 mM $MgCl_2$, pH 7.2), 50 μL of $D_2O$, 103 μL of 0.1 M CTP, and 247 μL of $H_2O$. This solution was adjusted to pH 7.1 with NaOH and mixed with 100 μL of synthetase solution [2.1 mg/mL, 4.6 μmol/(min·mg)] to give a total volume of 0.5 mL. Spectra of the pH 7.1 reaction mixture were recorded at 23 °C.

*Rate of Anomerization of α-NeuAc at pD 6.9.* A solution of *N*-acetylneuraminosyllactose (5.6 mg, 18 mM), $MgSO_4$ (1.2 mg, 20 mM), and $CD_3COOD$ (1.5 μL, 50 mM) 500 μL of $D_2O$ was adjusted to pD 5.4 with NaOD and treated with 1.5 unit of neuraminidase at 34 °C for 12 min [complete release of NeuAc was confirmed by NMR (Friebolin et al., 1980)]. This solution was cooled to 23 °C and adjusted to pD 6.9 by addition of 1 M Tris acetate-$d_3$ (56 μL, pD 7.9), and $^1$H NMR spectra were acquired at various times. The rate equation for the approach of a two component system to equilibrium was fitted to the intensities of the β-H3eq peak (δ 1.9 ppm) and the α-H3eq peak (2.5 ppm) to obtain the rate of conversion from the α-anomer to the β-anomer.

*Inhibition of CMP–NeuAc Synthetase Activity.* CMP–*N*-acetylneuraminic acid synthetase activity was measured by modification of methods described previously (Kean & Roseman, 1966; Vann et al., 1987). Assay mixtures contained 3 mM NeuAc and 2.5 mM substrate analogue. The background contributed by the analogue in the absence of neuraminic acid was subtracted out.

*Initial Velocity Measurements.* All enzyme rate studies were carried out using a 5 × 5 matrix of substrate concentrations at 37 °C. A typical assay contained CTP (0.13–2.00 mM), NeuAc (0.6–10.0 mM), buffer (250 mM bicine, 20 mM $MgCl_2$, and 0.4 mM dithiothreitol at pH 9.3), and the CMP–NeuAc synthetase solution; the final volume was 250 μL. Reactions were initiated by addition of enzyme and terminated after 30 min by reducing NeuAc with 50 μL of 1.6 M sodium borohydride. Excess sodium borohydride was removed by reaction with 50 μL of 20 N phosphoric acid at 0 °C. The product CMP–NeuAc was hydrolyzed to CMP and NeuAc by allowing the latter mixture to stand at 37 °C for 10 min. Reaction progress was measured by spectrophotometrically determining released NeuAc by either the thiobarbituric acid assay (Kean & Roseman, 1966; Vann et al., 1987) or the acidic ninhydrin assay described (Yao & Ubuka, 1987). *N*-Acetylneuraminic acid was used as the standard for both assays.

*Data Analysis.* The initial rate data were analyzed using the ENZFITTER program (copyright 1987 by Robin J. Leatherbarrow, distributed by Elsevier-Biosoft] with the basic Michaelis–Menten equation:

$$v = V^{app}[S_1]/(K_{S1}{}^{app} + [S_1])$$

where $[S_2]$ is held constant; $v$ is the initial rate, $V^{app}$ is the

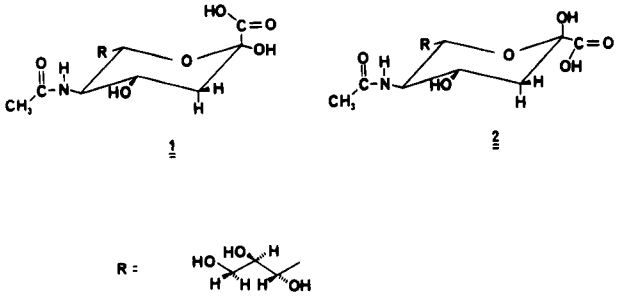

FIGURE 1: Structure of $\alpha$-*N*-acetylneuraminic acid (1) and $\beta$-*N*-acetylneuraminic acid (2).

apparent limiting rate at $[S_2]$, $K_{S1}^{app}$ is the apparent Michaelis constant for substrate 1 at $[S_2]$, $[S_1]$ is the molar concentration of substrate 1, and $[S_2]$ is the molar concentration of substrate 2. The coefficients of the lines shown in double-reciprocal plots were determined from the apparent values of the kinetic constants calculated by this program, where intercept = $1/V^{app}$ and slope = $K_{S1}^{app}/V^{app}$.

Data derived from the $^{13}C$ NMR spectrum with respect to the percent composition of reaction mixtures versus time were also analyzed using the ENZFITTER program. The area of the peaks for the $^{13}C$ NMR signals were considered to be a reasonable approximation of the composition because of the similarity of the environments of the carbons and the equilibrium composition of NeuAc solutions determine by $^{13}C$ NMR is similar to that reported from $^1H$ NMR results (Vliegenhart et al., 1982; Friebolin et al., 1980). Data regarding formation of CMP–NeuAc were fit to

$$N = A_{inf}[1-e^{(-kt)}]$$

where $N$ is the percent of CMP–NeuAc in the reaction mixture, $A_{inf}$ is the percent of CMP–NeuAc at infinity, $k$ is the time constant for the reaction in $min^{-1}$, and $t$ is the time after initiation in minutes.

Data regarding consumption of $\beta$-NeuAc were fit to

$$N = B_0e^{(-kt)} + B_{inf}$$

where $N$ is the percent of $\beta$-NeuAc in the reaction mixture, $B_0$ is a constant ($B_0 + B_{inf} = B_{initial}$ at $t = 0$ min), $k$ is the time constant for the reaction in $min^{-1}$, $t$ is the time after initiation in minutes, and $B_{inf}$ is the percent of $\beta$-NeuAc at infinity.

Data for the mutarotation of NeuAc at pD 6.9 were fit to

$$A = [A_o/(k_\alpha + k_\beta)][k_\alpha + k_\beta e^{(k_\alpha+k_\beta)t}]$$

where $A$ is the intensity of the $\alpha$-anomeric signal at time $t$, $A_o$ is the intensity at time zero, $k_\alpha$ is the rate of conversion of $\alpha$ to $\beta$, and $k_\beta$ is the rate of conversion of $\beta$ to $\alpha$. $k_\beta$ was initially estimated from the equilibrium constant, $K_{eq} = k_\beta/k_\alpha = 11.5$ (Friebolin et al., 1980) and was iterated to obtain the best fit.

## Results and Discussion

*N*-Acetylneuraminic acid (NeuAc) occurs predominantly as a six-member ring in the $^2C_5$ conformation (Vliegenthart et al., 1982). The $\alpha$- and $\beta$-isomers possess equatorial and axial anomeric hydroxyl groups, respectively (Figure 1), and exist in solution as a mixture of 7% $\alpha$-NeuAc and 93% $\beta$-NeuAc. In order to determine whether CMP–NeuAc synthetase exhibits any anomeric preference towards NeuAc, [2-$^{13}$C]NeuAc was prepared by enzymatic synthesis from 2-acetamido-2-deoxy-D-mannose and sodium [2-$^{13}$C]pyruvate using *N*-acetylneuraminic acid aldolase. $^{13}C$ NMR spectra of [2-$^{13}$C]NeuAc were recorded only for the region where the resonances due to C-2 were expected. This resonance for the $\alpha$-anomer was recorded at 98.9 ppm, with the $\beta$-anomer at 98.2 ppm. No indication of other ring forms was detected in these investigations. Reaction mixtures were prepared containing [2-$^{13}$C]NeuAc, $Mg^{2+}$, and CTP under conditions used for the colorimetric assay. Figure 2 shows the $^{13}C$ NMR spectra of a reaction mixture at three time points after the addition of CMP–NeuAc synthetase. The appearance and growth of a new resonance at 101.8 ppm was dependent upon the addition of $Mg^{2+}$ and enzyme and thus confirms its association with the formation of CMP–NeuAc. The multiplicity of the anomeric signal for CMP–NeuAc at 101.8 ppm observed in Figure 2 is the result of a two-bond coupling between the $^{13}C$-labeled anomeric carbon atom and the $^{31}P$ atom of the phosphate group.

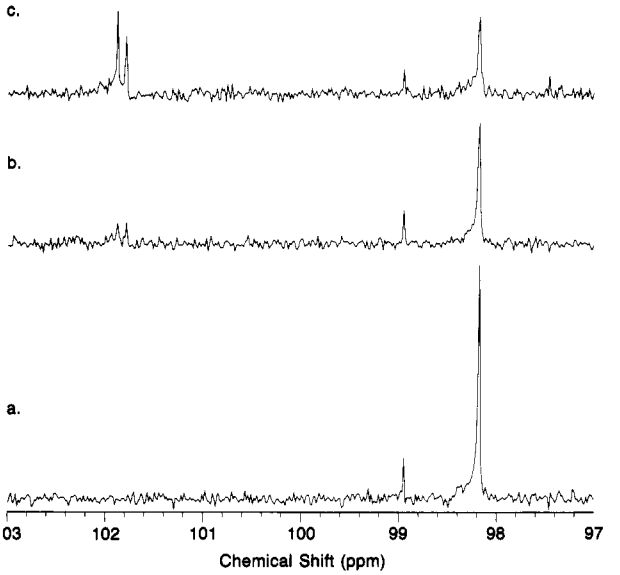


FIGURE 2: $^{13}C$ NMR Spectrum of the formation of CMP–NeuAc in the presence of CTP and [2-$^{13}$C]*N*-acetylneuraminic acid at various times after addition of CMP–NeuAc synthetase. [2-$^{13}$C]NeuAc (3.26 mg, 10.5 $\mu$mol) was dissolved in 490 $\mu$L of buffer solution (43 mM Tris, 35 mM $MgCl_2$, pH 8.5) and transferred to a 5-mm NMR sample tube. Deuterium oxide (100 $\mu$L, MSD Isotopes, "100 atom %") and 105 $\mu$L of 0.1 M CTP was added. The reaction was initiated by adding 100 $\mu$L of CMP–NeuAc synthetase solution, final volume 795 $\mu$L. The time between enzyme addition and the middle of data acquisition was 0, 32, and 211 min for spectra a, b, and c, respectively.

The kinetics of the conversion of NeuAc to CMP–NeuAc were thus followed by monitoring the fate of the anomeric carbon signal. The percent composition of anomeric species in the reaction mixture was monitored over time by measuring the areas under the respective resonance signals. This result is shown in Figure 3. It is quite apparent from inspection that the rate of formation of CMP–NeuAc is equal to the apparent rate of consumption of $\beta$-NeuAc. In fact, the exponential rates of formation and consumption were determined by nonlinear regression to be equivalent, 0.0075 and 0.0076 $min^{-1}$ respectively. The likely, although not the only, explanation for these results is that CMP–NeuAc synthetase exhibits specificity for $\beta$-NeuAc, the major anomer in solution. Preference for the $\alpha$-anomer would have been expected to result in the disappearance of its resonance signal from the $^{13}C$ NMR spectrum shortly after addition of CMP–NeuAc synthetase; indeed, Kohlbrenner and Fesik (1985) observed just this phenomenon when they studied the substrate specificity of CMP–KDO synthetase. They found that CMP–KDO synthetase clearly preferred $\beta$-KDO, the minor anomer in solution, and its resonance signal disappeared shortly after initiation of the reaction.

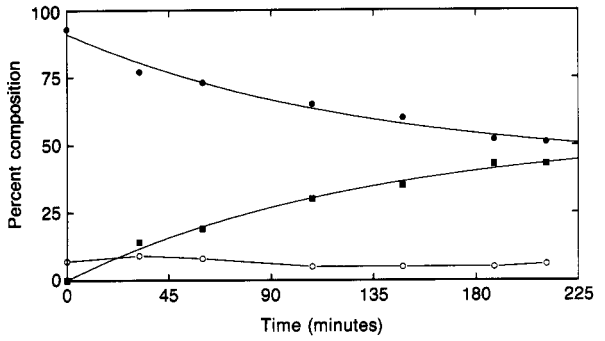


FIGURE 3: Percent composition of β-NeuAc (●), α-NeuAc (○), and CMP-NeuAc (■) versus time after addition of CMP–NeuAc synthetase. Values were estimated by integration of the resonance signals of the anomeric carbon atoms of the appropriate species at 0, 32, 60, 108, 148, 188, and 211 min after addition of CMP–NeuAc synthetase solution.

One other possible explanation of the data is that the α-anomer is being consumed by the enzyme and the observed decrease in β-anomer is due to a rapid mutarotation relative to the disappearance of the α-anomer. Friebolin et al. (1980) found the mutarotation rate for β-NeuAc to α-NeuAc to be slow at pH 5.4 ($k_\beta = 1.1 \times 10^{-5}$ s$^{-1}$). The rate of mutarotation as determined in our experiments at pH 8.5 was too rapid to be measured. Thus, in order to address the problem of rapid mutarotation, the enzymatic reaction was measured at pH 7.1 where the rate of mutarotation from the β to α anomer should be slower than the formation of product. The rate of conversion of the α to the β anomer ($k_\alpha$) was determined at pD 6.9 from the $^1$H NMR spectrum of neuraminidase-released α-NeuAc by the method of Friebolin et al. (1980). The rate constants derived from the appearance of the β-H3eq peak were $k_\alpha = 0.013$ min$^{-1}$ and $k_\beta = 0.0010$ min$^{-1}$ and from disappearance of the α-H3eq $k_\alpha = 0.019$ min$^{-1}$ and $k_\beta = 0.0014$ min$^{-1}$.

The rate of formation of [2-$^{13}$C]CMP–NeuAc was determined at pH 7.1 as described above for the measurements at pH 8.5 in the presence of a higher concentration of enzyme and substrates. Rate constants derived for the formation of CMP–NeuAc and the consumption of β-NeuAc were 0.020 and 0.026 min$^{-1}$, respectively. Under these conditions at pH 7.1, the enzymatic rate is faster that the rate of mutarotation. As was observed at pH 8.5, the rate of disappearance of β-[2-$^{13}$C]NeuAc is equivalent to that of the rate of formation of [2-$^{13}$C]CMP–NeuAc. The results are consistent with the preferential consumption of the β-anomer and not the α-anomer.

The importance of the anomeric center in the interaction of NeuAc with the *E. coli* enzyme was further studied using NeuAc analogues. The β-methyl ketoside of NeuAc is a competitive inhibitor of mammalian CMP–NeuAc synthetase (Petri et al., 1989). The β-methyl ketoside of NeuAc is a poor inhibitor of *E. coli* CMP–NeuAc synthetase, exhibiting less than 20% inhibition at NeuAc concentrations at or below the $K_m$. Schmid et al. (1988) reported a $K_i$ of 2.5 mM for this analogue. No inhibition was observed with the 2,3-unsaturated analogue, 2,3-dehydro-*N*-acetylneuraminic acid. This analogue is a potent inhibitor of neuraminidases which utilize the α-anomer of NeuAc (Meindl & Tuppy, 1969).

The 2,3-unsaturated analogue 2,3-dehydro-*N*-acetylneuraminic acid was converted to β-2-deoxy-*N*-acetylneuraminic acid by catalytic hydrogenation. The peracetylated intermediate gave spectral properties identical with results obtained by Schmid et al. (1988). The β orientation of the proton at carbon 2 (C-2) was confirmed by the magnitude of the H2–H3

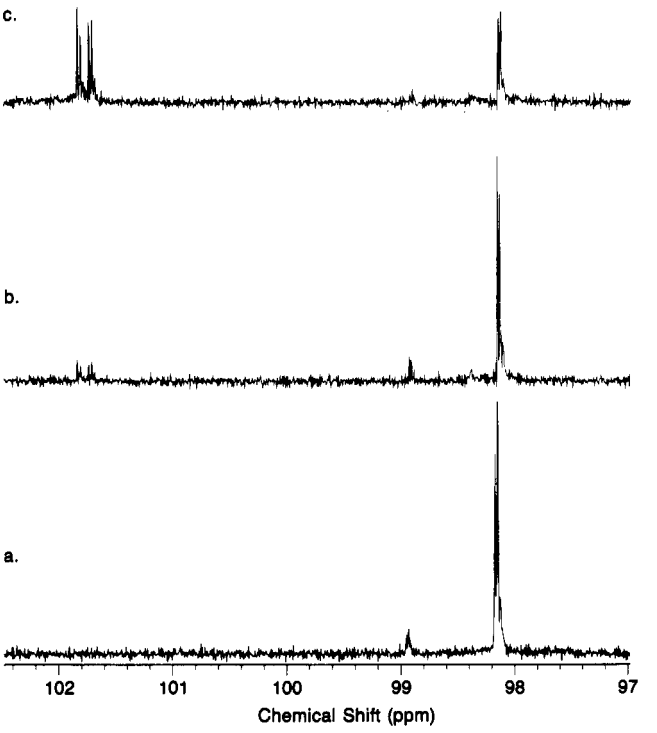


FIGURE 4: $^{13}$C NMR spectrum of the formation of CMP–NeuAc in the presence of CTP and [2-$^{13}$C; (50 atom %)$^{18}$O]*N*-acetylneuraminic acid at various times after addition of CMP–NeuAc synthetase. A solution of CTP (100 μL, 0.085 M, pH 7) and 100 μL of CMP–NeuAc synthetase solution was added to the [2-$^{13}$C,(50%)$^{18}$O]NeuAc solution whose preparation is described under Materials and Methods. The time between enzyme addition and the middle of data acquisition was 0, 23, and 285 min for spectra a, b, and c, respectively.

coupling constant, $J = 12$. This compound, β-2-deoxy-*N*-acetylneuraminic acid, is a weak inhibitor of CMP–NeuAc synthetase (15% inhibition). Thus, the hydroxyl at C-2 plays an important role in the interaction of substrate with the *E. coli* enzyme since analogues which either lack or have a substituted anomeric oxygen are poor enzyme inhibitors. The mammalian CMP–NeuAc synthetase is not inhibited by β-2-deoxy-*N*-acetylneuraminic acid (Schmid et al., 1988; Petrie et al., 1989) nor does the β-glycosyl fluoride inhibit. Thus both the bacterial and mammalian CMP–NeuAc synthetase require the anomeric oxygen for recognition although not to the same extent.

The fate of the anomeric oxygen atom was ascertained by observing the $^{13}$C NMR spectrum over time with a mixture of [2-$^{13}$C]NeuAc and [2-$^{13}$C,$^{18}$O]NeuAc as substrate (Figure 4). The latter was prepared by partial $^{18}$O exchange with [$^{18}$O]water at pH 9. Each of the resonances of the two anomers appears as a doublet. The multiplicity results from the effect of the $^{18}$O-isotope shift. A roughly equal mixture of $^{18}$O-substituted and unsubstituted NeuAc gives two signals with the $^{18}$O-substituted anomeric carbon shifted upfield by 1.5 Hz (0.02 ppm). After addition of CMP–NeuAc synthetase, a pair of doublets appears and their intensity increases steadily as the reaction progresses. This pattern results from coupling between the $^{13}$C atom at the anomeric center and the $^{31}$P atom of the phosphoryl group, as previously observed, and the existence of both $^{16}$O- and $^{18}$O-substituted CMP–NeuAc. The implication of this observation is that the oxygen atom bonded to the anomeric carbon of NeuAc is retained in a bridging position between the CMP and NeuAc moieties. This would support a mechanism in which O-2 is the nucleophile in an $S_N2$-type displacement process since loss of this oxygen to the solvent would have resulted in the appearance of only

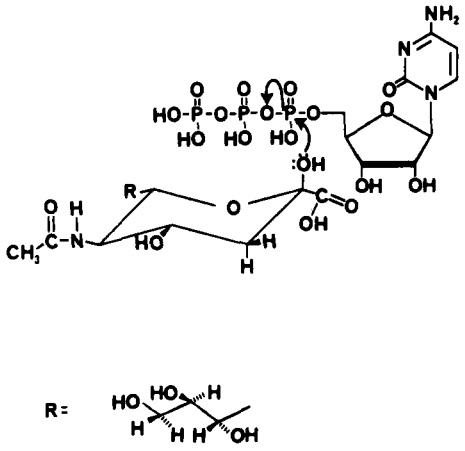

FIGURE 5: Enzyme-mediated $S_N2$ reaction of *N*-acetylneuraminic acid with cytidine triphosphate resulting in the formation of CMP–*N*-acetylneuraminic acid and pyrophosphate.

a single doublet in the $^{13}C$ NMR spectrum. This parallels the mechanism that has been discerned for CMP–KDO synthetase in regards to KDO (Kohlbrenner et al., 1987). CMP–NeuAc occurs in nature as the $\beta$-anomer (Vliegenthart et al., 1982). The retention of the anomeric oxygen supports the idea that *E. coli* CMP–NeuAc synthetase consumes the $\beta$-anomer on NeuAc. A loss of anomeric oxygen to solvent would be more compatible with a mechanism utilizing either anomer.

The steady-state kinetic mechanism was also investigated. The Lineweaver–Burk transformation of the initial rate versus CTP concentration yielded converging lines. The converging lines imply a sequential mechanism involving a ternary complex of the enzyme, CTP, and NeuAc. A substituted-enzyme mechanism, often referred to as a ping-pong mechanism, would have been expected to yield a set of parallel lines. The substituted-enzyme mechanism involves a nucleophilic moiety in the active site of the enzyme displacing a leaving group on the first bound substrate resulting in formation of the first product, the leaving group, and a covalent substrate–enzyme intermediate. By contrast, a mechanism involving a ternary complex would involve a direct transfer between the two bound substrates.

While it is possible that the enzyme may use both substrates, it is difficult to imagine how the $\alpha$-anomer could be used to form $\beta$-CMP–NeuAc with the conservation of the anomeric oxygen without some sort of mutarotation on the enzyme. We believe that we have chosen the simplest mechanism to explain all of the observations which are (a) the apparent rate of product formation equals the disappearance of the $\beta$-anomer, (b) at pH 7.1 the rate of anomerization is slow relative to product formation, (c) no apparent loss of anomeric oxygen, (d) a non-ping-pong-type mechanism, and (e) the product is in the $\beta$ configuration.

Our interpretation of the results of the NMR and steady-state kinetics experiments in this report is summarized in the mechanism illustrated in Figure 5. CMP–NeuAc synthetase catalyzes the direct displacement of pyrophosphate from CTP by the anomeric oxygen atom of NeuAc yielding CMP–NeuAc.

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# Reduction of the Small Subunit of *Escherichia coli* Ribonucleotide Reductase by Hydrazines and Hydroxylamines

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ABSTRACT: Each polypeptide chain of protein R2, the small subunit of ribonucleotide reductase from *Escherichia coli*, contains a stable tyrosyl radical and an antiferromagnetically coupled diferric center. Recent crystallographic studies [Nordlund, P., Eklund, H., & Sjöberg, B.-M. (1990) *Nature 345*, 593-598] have shown that both the radical and the diiron site are deeply buried inside the protein and thus strongly support the hypothesis of long-range electron-transfer processes within protein R2. This study shows that monosubstituted hydrazines and hydroxylamines are able to reduce the tyrosyl radical and the ferric ions, under anaerobic conditions. It allows characterization of the site from which those compounds transfer their electrons to the iron/radical center. The efficiency of any given reducing agent is not solely governed by its redox potential but also by its size, its charge, and its hydrophobicity. We suggest, as a possible alternative to the long-range electron-transfer hypothesis, that conformational flexibility of the polypeptide chain might exist in solution and allow small molecules to penetrate the protein and react with the iron/radical center. This study also shows that two reduction mechanisms are possible, depending on which center, the radical or the metal, is reduced first. Full reduction of protein R2 yields reduced R2, characterized by a normal tyrosine residue and a diferrous center. Both the radical and the diferric center are regenerated from reduced R2 by reaction with oxygen, while only the diferric center is formed by reaction with hydrogen peroxide.

Ribonucleotide reductase is a key enzyme for all living organisms. It provides the deoxyribonucleotides required for the synthesis of deoxyribonucleic acid (DNA)[1] (Lammers & Follman, 1983; Reichard, 1988; Stubbe, 1990). The enzyme from *Escherichia coli* contains two homodimeric proteins that can be separated during purification. Each polypeptide chain of the small protein, named protein R2, contains a stable radical located on tyrosine-122 (Reichard & Ehrenberg, 1983; Larsson & Sjöberg et al., 1986) as well as a binuclear iron center, in which the Fe(III) ions are antiferromagnetically coupled by a $\mu$-oxo bridge (Petersson et al., 1980; Sjöberg et al., 1982). Protein R2 has been crystallized, and a refined three-dimensional structure is now available (Nordlund et al., 1990).

The radical is absolutely required for enzyme activity and is thus believed to participate in the activation of the sugar moiety during ribonucleotide reduction (Stubbe, 1989). *Escherichia coli* contains an enzyme system that introduces the radical into the protein (Barlow et al., 1983; Fontecave et al., 1987a). It thus provides the cell with the ability to regulate the activity of ribonucleotide reductase through the amount of tyrosyl radical present in R2.

The radical is found buried within the protein (Nordlund et al., 1990). However, it is known to react with a large number of radical scavengers, antioxidants, and reductants (Reichard & Ehrenberg, 1983; Lammers & Follman, 1983). This has been the basis of numerous studies on the inhibition of ribonucleotide reductase. In particular, hydroxyurea and hydroxylamine are excellent scavengers of the tyrosyl radical (Ehrenberg & Reichard, 1972; Atkin et al., 1973). In contrast, the iron center was originally thought to be chemically inert. Iron is tightly bound to the polypeptide chain, and preparation of apoR2, the apoprotein, requires extensive dialysis against a very strong iron chelator (Atkin et al., 1973).

A few recent reports have shown that electrons could be transferred to the diferric site, which results in the formation of reduced R2 with a diferrous center + a normal tyrosine residue. Therefore, protein R2 can be prepared in three redox states: (i) as active R2, the fully oxidized state, with a binuclear Fe(III) center + a tyrosyl radical; (ii) as metR2, with a Fe(III) center + a normal tyrosine-122; (iii) as reduced R2. The reduction of the iron center can be chemically achieved by reaction with dithionite in the presence of catalytic amounts of a redox mediator such as viologens (Sahlin et al., 1989), with dithiothreitol at alkaline pH (Fontecave et al., 1990a), and with diimide (Gerez et al., 1991). The bacterial radical-introducing enzyme has also been shown to function as a protein R2–diferric reductase and might be the physiological reductant (Fontecave et al., 1987a, 1989). The formation of reduced R2 is a key step during enzymatic activation of protein R2 since the tyrosyl radical can only be generated during the reoxidation of the ferrous center by oxygen (Fontecave et al., 1987a,b).

In this study, we have tried to establish the stereoelectronic parameters that control the reactivity of the iron center of protein R2. Since the only reaction of ferric iron in ribo-

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[1] Abbreviations: DNA, deoxyribonucleic acid; DTT, dithiothreitol; EPR, electronic paramagnetic resonance; Tris, tris(hydroxymethyl)-aminomethane.

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