Why Is CMP-Ketodeoxyoctonate Highly Unstable?†

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ABSTRACT: CMP-ketodeoxyoctonate (CMP-KDO) and analogs, including CMP-5-deoxy-5-fluoro-KDO, CMP-5-deoxy-KDO, and CMP-5-*epi*-KDO, were prepared from CTP and the corresponding KDO sugars catalyzed by CMP-KDO synthetase. These analogs were found to be much more stable than CMP-KDO ($t_{1/2}$ = 0.57 h) yet less stable than CMP-sialic acid ($t_{1/2}$ = 151 h). Fluorination at the 5-position of CMP-KDO has a 200-fold enhanced stability compared to the 156-fold enhancement for the 3*R*-fluoro analog, probably due to the loss of H-bonding interactions (for the 5-F derivative) and the cause of remote inductive effect (for the 3- and the 5-F analogs) on the glycosidic cleavage. Hydrolysis of CMP-KDO is perhaps facilitated by an intramolecular hydrogen bond from the 5-OH group with the phosphate oxygen as demonstrated by the 3−5-fold enhanced stability of CMP-5-*epi*-KDO and CMP-5-deoxy-KDO compared to CMP-KDO and by molecular modeling studies of water-solvated CMP-KDO. Hydrolysis of CMP-KDO also was found to be subject to a substantial solvent isotope effect (k_H/k_D = 2.7), which is significantly different from the reported solvent isotope effect for the hydrolysis of sialylglycosides (k_H/k_D = 0.86) and dependent on both buffer and magnesium ion concentrations. Considering these results and molecular modeling studies, it is proposed that the hydrolysis of CMP-KDO under neutral conditions proceeds through a glycosidic cleavage which occurs at the electronically favorable twist-boat conformation, facilitated by intramolecular H-bonding interaction of the 4-, 5- and 7- (or 8-) OH groups and the phosphate oxygen and by the leaving group magnesium ion complexation.

Phosphate diesters are very stable in aqueous solution under neutral conditions. The half-life of the phosphate diester linkage in DNA, for example, has been estimated to be 200 million years at pH 7 (Chin et al., 1989). The phosphodiesters of sugar nucleotides, activated monosaccharide substrates for the Leloir pathway glycosyltransferases (Leloir, 1971), are, however, very unstable. Among these sugar nucleotides, CMP-KDO (cytidine 5′-monophospho-3-deoxy-D-*manno*-2-octulosonic acid, CMP-ketodeoxyoctonate) is surprisingly labile with a half-life of 34 min (at 25 °C, in 0.1 M Tris-HCl buffer, pH 7.5, and 20 mM $MgCl_2$) (Sugai et al., 1995). In fact, this is the reason that CMP-KDO has never been synthesized chemically or isolated as the product of the CMP-KDO synthetase reaction. CMP-KDO is the key intermediate in the biosynthesis of bacterial lipopolysaccharides and its formation is the rate-determining step in the enzymatic process (Osborn, 1979; Ray et al., 1981; Unger, 1981), indicative of the importance of CMP-KDO synthetase as a target for development of antibacterial agents.

Recently, we have reported that CMP-5-deoxy-5-fluoro-KDO (CMP-5-FKDO) prepared by CMP-KDO synthetase was unexpectedly stable ($t_{1/2}$ = 4.7 days) and was subsequently isolated for structural determination (Sugai et al., 1995). From the significant difference in the stability between CMP-KDO and CMP-5-FKDO, the 5-OH of CMP-KDO was proposed to play an important role in the decomposition. CMP-KDO hydrolysis has been proposed to proceed through a twist-boat conformation (Scheme 1) with the 5-OH group H-bonding interaction with the glycosidic *exo*-oxygen and the 7-OH (or 8-OH) H-bonding interaction with the negative charge of the phosphate group (Sugai et al., 1995). In the chair form, the 7- and 8-OH groups would also have a close contact with the phosphate negative charge, but the 5-OH is far away from the glycosidic *exo*-oxygen.

Although the twist-boat form of CMP-KDO was not observed in solution by NMR, KDO does exist as a mixture of chair and twist-boat forms, as 9.5% of KDO in aqueous solution exists as a 1,5-lactone (Sugai et al., 1993). Furthermore, the large coupling constant between C-1 and P in the KDO moiety of CMP-KDO ($^3J_{P-C1}$ = 6.9 Hz) indicates that a *trans* dihedral angle (*endo* phosphate) exists between the phosphate group and the carboxylic acid group in which the phosphate group is in contact with the 5- and potentially 7- or 8-OH groups (Kohlbrenner & Fesik, 1985). The related compound, KDO-β-2-phosphate, has been reported to have a similar three-bond coupling constant ($^3J_{P-C1}$ = 7.95 Hz) (Baasov & Kohen, 1995). These intramolecular H-bonding interactions are not possible in CMP-sialic acid ($^3J_{P-C1}$ = 1 Hz), in which the phosphate group is extending away from the sialic acid ring in a *gauche* orientation (*exo* phosphate) with respect to the protons at C-3 (Haverkamp et al., 1979).

In this paper, the enzymatic synthesis and characterization of two novel CMP-KDO analogs (5-*epi* and 3*R*-fluoro) are described. These two analogs in addition to CMP-KDO and its 5-fluoro- and 5-deoxy analogs were used to probe the role of hydroxyl topography and electronic requirements of

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[1] Abbreviations: CMP-KDO, cytidine 5′-monophospho-3-deoxy-D-manno-2-octulosonic acid, CMP-ketodeoxyoctonate; KDO, ketodeoxyoctonate, 3-deoxy-D-manno-2-octulosonic acid; CMP-5FKDO, cytidine 5′-monophospho-3,5-dideoxy-5-fluoro-D-*manno*-2-octulosonic acid (5-fluoro derivative of CMP-KDO); CMP3FKDO, cytidine 5′-monophospho-3*R*-3-deoxy-3-fluoro-D-*manno*-2-octulosonic acid (3-fluoro derivative of CMP-KDO); UDP-GlcNAc, uridine diphosphate-α-D-*N*-acetylglucosamine; GDP-fuc, guanosine diphosphate-β-L-fucose.

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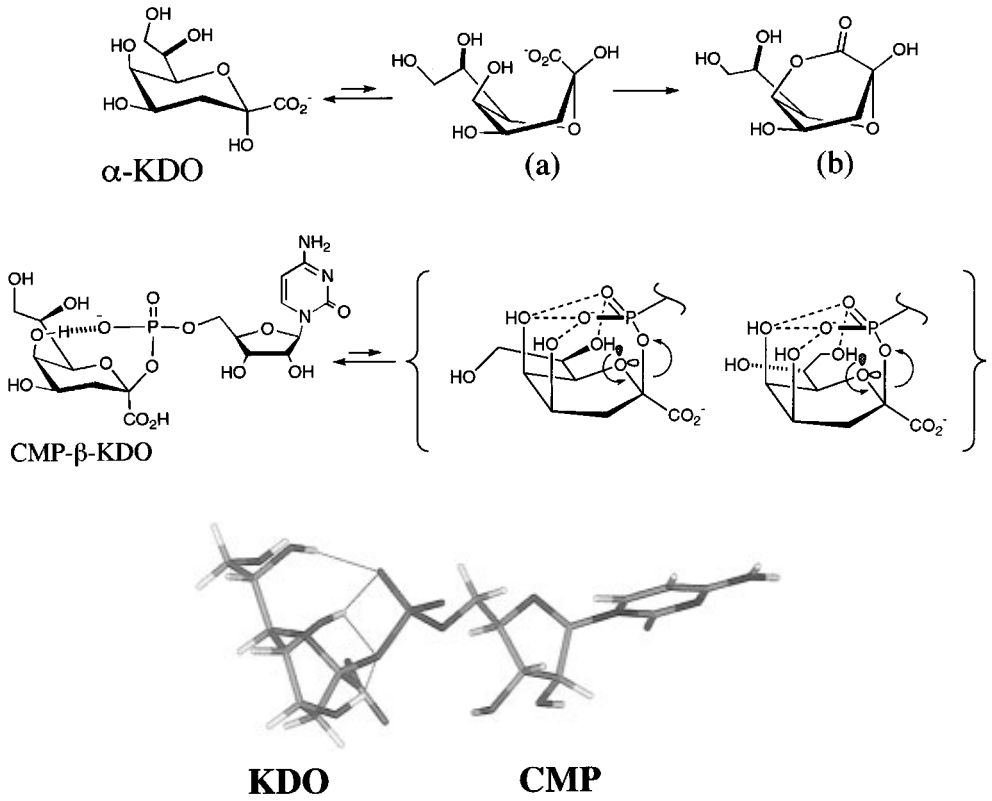
Scheme 1: Proposed Mechanism of CMP-KDO Hydrolysis[a]



[a] Formation of the α-KDO 1,5 lactone (b) was observed in aqueous solution. A similar conformation (twist boat) is proposed for CMP-*β*-KDO in which the phosphate group interacts with the 4, 5 and 7 (or 8) hydroxyl groups to facilitate glycosidic cleavage (see the structure generated by computer modeling).

the hydrolysis reaction. CMP-KDO instability was investigated with studies of the effects of solvent, buffer, pH, and magnesium on the hydrolysis rate including the identification of a large solvent isotope effect. These studies in addition to molecular modeling of solvated CMP-KDO were performed to provide insight into the mechanism of CMP-KDO hydrolysis.

MATERIALS AND METHODS

We have cloned and overexpressed CMP-KDO synthetase (EC 2.7.7.38) in *Escherichia coli*, which was published previously (Sugai et al., 1995). The protein pellet obtained after ammonium sulfate precipitation was used without further purification. All the reagents and chemicals were purchased from Sigma including inorganic pyrophosphatase and alkaline phosphatase unless otherwise specified. ^{1}H and ^{19}F NMR were obtained with a Bruker AMX-500 spectrometer. ^{19}F NMR spectra were referenced externally to trifluoroacetic acid.

Synthesis of CMP-KDO and Analogs. The preparation of CMP-KDO and analogs was performed as follows: A solution of 200 mM Tris-HCl buffer (pH 9.0, 5.3 mL) containing 20 mM $MgCl_2$, 0.2 mM dithiothreitol, CTP (Sigma C-9274, disodium salt dihydrate, 84.8 mg, 0.176 mmol, 30 mM final concentration), and KDO or a KDO analog (0.117 mmol, 20 mM final concentration) was first prepared. To this was added inorganic pyrophosphatase (Sigma I-4503, 100 units/mL solution, 30 mL, 3 units) and CMP-KDO synthetase (300 mL, 15 units). The mixture was stirred at 25 °C under argon. Formation of product was monitored by NMR. The H_{3ax} and H_{3eq} ^{1}H NMR signals were used to identify the presence of CMP-KDO (or CMP-5-deoxy-KDO), which have chemical shifts of 2.15 and 2.50 ppm, distinct from the H_{3ax} and H_{3eq} of KDO (1.90 and 1.81 ppm). ^{19}F NMR was used to follow the formation of CMP-3R-FKDO from 3R-FKDO, which have signals at about −209.8 and −209.4 ppm, respectively. The reaction time was dependent on the specificity of substrates. A maximal amount of CMP-KDO was produced after a reaction time of 40−50 min.

CMP-KDO and analogs were purified as follows: The precipitated magnesium phosphate was removed by centrifugation. For stability studies involving CMP-KDO in the absense of $MgCl_2$, Chelex 100 resin (NH_4^+ form) was used to remove the magnesium ions after the reaction was stopped (Belnius & Raetz, 1992). The supernatant was further centrifuged through on Ultrafree-MC centrifuge tube (Nihon Millipore Kogyo, K. K., exclusion limit 10 000 MW) to remove the proteins. The resulting solution was lyophilized and dissolved in 100 mM Tris buffer (pH 7.5 containing 20 mM $MgCl_2$) for the stability study. The NMR assignment of CMP-5-F-KDO was consistent with the literature (Sugai et al., 1995) but CMP-KDO and its other analogs were not fully characterized because of their relative instability.

Stability Studies of CMP-KDO and Analogs. CMP-KDO and analogs were prepared by the CMP-KDO synthetase procedure described above. As in the synthetic procedure, the H_{3ax} and H_{3eq} ^{1}H NMR signals were used to identify the presence of CMP-KDO and CMP-5-deoxy-KDO and in this case used to follow their decomposition. These have chemical shifts of 2.15 and 2.50 ppm, which are distinct from the H_{3ax} and H_{3eq} of KDO and 5-deoxy-KDO (1.90 and 1.81

ppm). ^{19}F NMR was used to follow the decomposition of CMP-3R-FKDO to 3R-FKDO, in which the ^{19}F signals appear at −209.8 and −209.4 ppm, respectively.

In agreement with the literature (Kajimoto et al., 1991; Kohlbrenner & Fesik, 1985; Kohlbrenner et al., 1987), CMP-KDO gradually decomposed to CMP and KDO, and the decomposition was a first-order reaction:

$$\frac{d[\text{CMP-KDO}]}{dt} = -kt \quad (1)$$

$$\ln([\text{CMP-KDO}]_t) = \ln([\text{CMP-KDO}]_0) - kt \quad (2)$$

The decomposition rate of CMP-KDO (or analogs) was determined on the basis of the remaining CMP-KDO (or analogs) in the mixture as determined by ^{1}H or ^{19}F NMR analysis, as shown in Figure 2, and the half-lives were calculated from the ^{1}H-NMR data (Figure 3).

Stability Study of Other Sugar Nucleotides. The sugar nucleotides investigated for their stability study in the text existed originally as sodium salts. GDP-Fuc was synthesized on the basis of the known procedure of our group, and the ^{1}H and ^{13}C NMR spectra of the desired product were identical to those of literature (Ichikawa et al., 1992). CMP-KDO was also prepared as mentioned in the same source. After CTP was almost consumed, the reaction solution was then passed through a column of Chelex 100 (Sigma Co., Na^+ form, 20 mL) to remove the magnesium ions. The resulting solution was lyophilized to a dry powder for use in the stability study.

A solution of 100 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$ and 20 mM sugar nucleotide was prepared and transferred into a NMR tube with a coaxial insert (for the external reference, D_2O). Each stability test was performed at 25 °C by ^{1}H NMR analysis. For GDP-Fuc, the H-8 of the nucleotidic base and CH_3 of the fucose (chemical shifts 8.0−8.1 ppm and 1.1−1.2 ppm, respectively) were used to determine decomposition rates. For GDP-Man, UDP-Gal, and UDP-GlcNAc, the protons of the base were used. CMP-NeuAc was analyzed in a manner similar to the case of CMP-KDO.

Computer Modeling. To better understand the extremely rapid rate at which CMP-KDO decomposes in water compared to other glycosylated nucleotides, a model of CMP-KDO was constructed using Insight/Discover (Biosym, San Diego, CA). The model was initially minimized using the cvff92 force field. The model was then soaked in a solvation shell of 114 water molecules and placed in a lattice cell of dimensions 20 by 15 by 15 Å. The model was subject to a dynamics run of 100 000 iterations at 300 K (cvff92), with a structure saved every 100 iterations, yielding a library of 1000 conformations. These were analyzed for consistent conformational motifs and H-bonding patterns.

RESULTS AND DISCUSSION

To gain more insights into the mechanism of CMP-KDO decomposition, several KDO analogs [including 5-deoxy (Sugai et al., 1993), 5-*epi* (Sugai et al., 1993), and *3R*-3-deoxy-3-fluoro-KDO (Kohen et al., 1993)] were tested as substrates for CMP-KDO synthetase. The reaction product of the KDO-8-phosphate synthase-catalyzed reaction of (*E*)-3-fluorophosphoenol pyruvate with D-arabinose 5-phosphate, 3*R*-FKDO-8-phosphate, was processed with alkaline phosphatase to yield 3*R*-FKDO (Kohen et al., 1993). These compounds were all substrates for CMP-KDO synthetase and the CMP products are all more stable than CMP-KDO (Table 1). The stability can be easily monitored by ^{19}F or ^{1}H NMR analysis (Figures 1 and 2), and the decompostion rates calculated from the integration of the appropriate resonances (Figure 3). These results indicate that the hydrolysis may involve a glycosidic cleavage (Scheme 1) which is facilitated by the intramolecular H-bonding interactions. The cleavage reaction may proceed with oxocarbonium character because the 3-F and 5-F derivatives should destabilize the transition state through an inductive effect, and in the case of the 5-F derivative, the 5-OH group is no longer available for H-bonding with the phosphate group (Bunton & Humeres, 1969; Bunton et al., 1958; Jencks, 1981; Sinnott, 1984; Withers et al., 1986, 1989). For example, the hydrolysis of glucose-α-1-phosphate is reported to be 60-fold faster than that of the 2-fluoro derivative and 9−15-fold faster than that of the 3-fluoro and 4-fluoro derivatives (Withers et al., 1986).

Table 1: Stability of CMP-KDO and Its Derivatives Determined from the Pseudo-First-Order Decomposition Kinetics

CMP derivatives	half-life (h)	relative stability
-KDO	0.57	1
-5-deoxy-KDO	2.5	4.0
-5-*epi* -KDO	1.7	3.0
-5-fluoro-KDO	114	200
-*3R*-fluoro-KDO	89	156

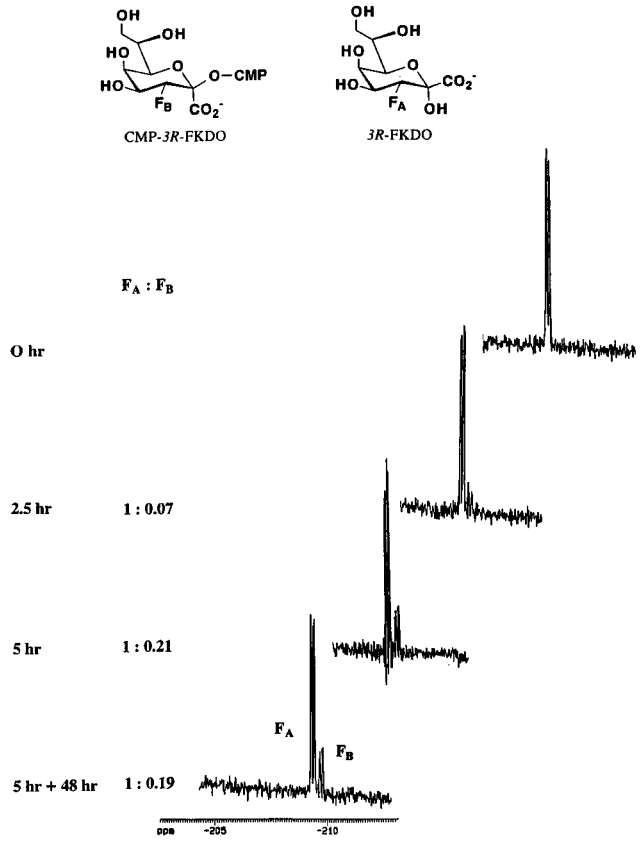


FIGURE 1: Stability of CMP-3*R*-FKDO monitored by ^{19}F NMR analysis. The top three traces are the spectra of the enzymatic reactions for 0, 2.5, and 5 h, respectively. The bottom trace is the spectrum taken another 48 h after the reaction was stopped. The ratio was determined on the basis of the integration of F_A for 3*R*-FKDO and F_B for CMP-3*R*-FKDO.

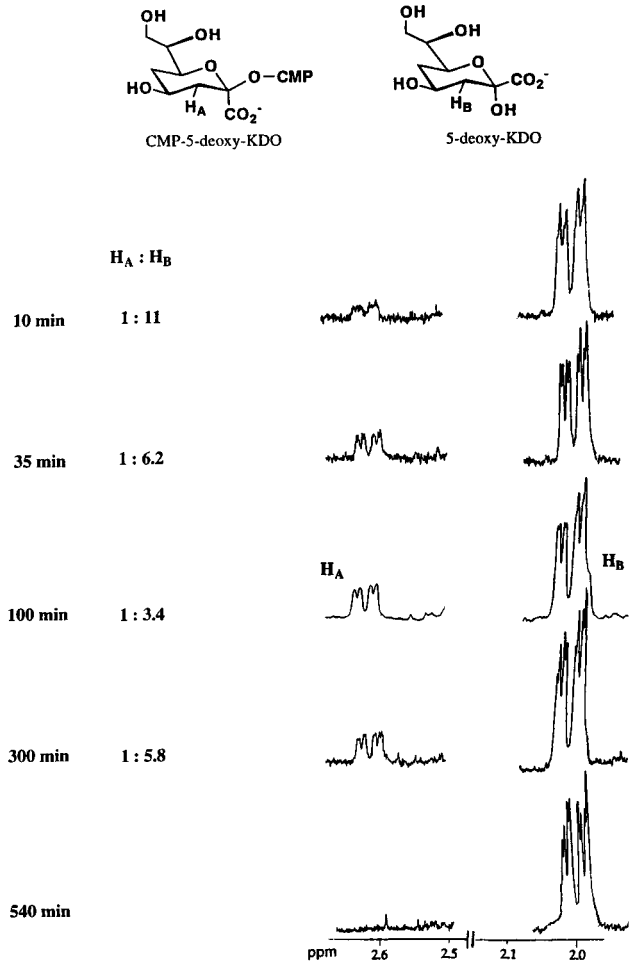


FIGURE 2: Stability of CMP-5-deoxy-KDO monitored by ^{1}H NMR analysis. CMP-5-deoxy-KDO has been generated by the CMP-KDO synthetase-catalyzed reaction before the study was started. The ratio was determined on the basis of the integration of H_A for CMP-5-deoxy-KDO and H_B for 5-deoxy-KDO.

For comparison, the hydrolysis rates of CMP-sialic acid, GDP-mannose, UDP-galactose, UDP-GlcNAc, and GDP-fucose were measured with NMR studies (Table 2). The hydrolysis of CMP-sialic acid was 265-fold slower than that of CMP-KDO. The other nucleotide sugars were much more stable, which precluded the determination of half-lives, and therefore were estimated to have a half-life much greater than 1 week (168 h). The half-life of GDP-fucose has been determined by another method to be 302 h (pH 6.0, 20 °C, 10 mM $MnCl_2$) and 3340 h in the absence of manganese ions (Murray et al., 1996). An interesting observation is that CMP-sialic acid is more stable than either CMP-5FKDO or CMP-3FKDO.

The mechanism of CMP-KDO hydrolysis was further investigated with a kinetic isotope effect experiment. A substantial normal deuterium solvent isotope effect ($k_{H_2O}/k_{D_2O} = 2.7$) on the decomposition of CMP-KDO was observed (Figure 4). This isotope effect is consistent with a mechanism involving general acid catalysis. Acetal hydrolysis typically proceeds via a specific acid catalysis, either by an A-1 or A-2 mechanism (Lowry & Richardson, 1987), which both have inverse solvent isotope effects ($k_{H_2O}/k_{D_2O} < 1$).(Fife, 1967) The hydrolysis of α-D-glucose-1-phosphate proceeds via an A-1 mechanism with an inverse solvent isotope ($k_{H_2O}/k_{D_2O} = 0.57$) (Bunton & Humeres, 1969). An inverse solvent kinetic isotope effect ($k_{H_2O}/k_{D_2O} = 0.86$) was observed in the hydrolysis of the sialyl glycoside monoanions ($-CO_2^-$) (Ashwell et al., 1992). The solvent kinetic isotope effect of the neutral sialylglycoside ($-CO_2H$) was determined to be 0.96, which was used to rule out intramolecular, general acid catalysis by the C-1 carboxylic acid (Ashwell et al., 1992). Formic acid-catalyzed hydrolysis of the acetal tetrahydropyranyl *p*-nitrophenyl ether is known to be a general acid-catalyzed reaction with a deuterium solvent isotope effect of 3.4, similar to the isotope effect observed for the hydrolysis of CMP-KDO (Fife & Brod, 1970). There are many examples of intramolecular carboxylic acid-catalyzed glycoside/acetal hydrolysis reactions (Capon, 1969; Capon et al., 1969; Piszkiewicz & Bruice, 1968; Wang & Withers, 1995). Capon and co-workers found that the intramolecular carboxylic acid-catalyzed hydrolysis of glycosides and acetals was general acid-catalyzed because they contained *suitably oriented* carboxylic groups and displayed significant normal isotope effects (Capon et al., 1969). These results are consistent with a C-1 carboxylic acid-catalyzed hydrolysis of CMP-KDO, especially at the pH lower than that of the carboxyl pK_a.

Increasing the concentration of phosphate buffer from 10 to 100 mM made CMP-KDO less stable, as observed in the half-life shift from 34 to 18 min, which is consistent with general acid catalysis. No buffer catalysis was detected in the hydrolysis of sialyl glycosides at both pH 2.69 and 6.75, which is expected for specific acid catalysis (Ashwell et al., 1992). During the stability study of CMP-KDO, hydrolysis was faster in the presence of $MgCl_2$ ($t_{1/2} = 34$ min) than in the absence of magnesium ions ($t_{1/2} = 65$ min), indicating that Mg^{2+} may act to neutralize the phosphate of the leaving group and thus facilitate the glycosidic cleavage (Scheme 1). Support for this argument was found in the detailed study of the metal ion-catalyzed hydrolysis of phosphate esters (Browne & Bruice, 1992; Chin, 1991; Chin & Kim, 1992; Dempcy & Bruice, 1994) and succinate esters (Fife & Przystas, 1982). The hydrolysis of GDP-fucose is accelerated 10-fold in the presence of 10 mM $MnCl_2$ (pH 6.0, 20 °C) (Murray et al., 1996).

A flat pH profile of CMP-KDO hydrolysis in the region between pH 7.5 and 8.5 was observed with increased hydrolysis rates at pH 7 and 9 in both Tris and phosphate buffers (Figure 5). This profile may be rationalized by the shifting of the hydrolysis reaction from an acid-catalyzed reaction to a base-catalyzed reaction. This effect was observed in the hydrolysis of both *o*-carboxyphenyl β-D-glucopyranoside (Piszkiewicz & Bruice, 1968) and *o*-nitrophenyl glucosides (Piszkiewicz & Bruice, 1967). Interestingly, CMP-KDO is hydrolyzed about 14% faster when 5% 1,4-dioxane is added to the solution.

To further investigate the origin of the instability, a molecular model of CMP-KDO was then constructed using the Insight/Discover program, minimized with the cvff92 force field, and subjected to 100 000 interations of molecular dynamics in a solvent shell of 114 water molecules. All of the structures obtained in the molecular modeling studies maintained a hydrogen bond between the 5-OH of CMP-KDO and one of the free oxygen atoms of the phosphate group, despite the constant availability of competing water molecules to accept this hydrogen bond, consistent with NMR measurements of a C1−P *anti* orientation about the glycosidic bond and unlike CMP-sialic acid, where such a hydrogen bond is not possible and a *gauche* conformation

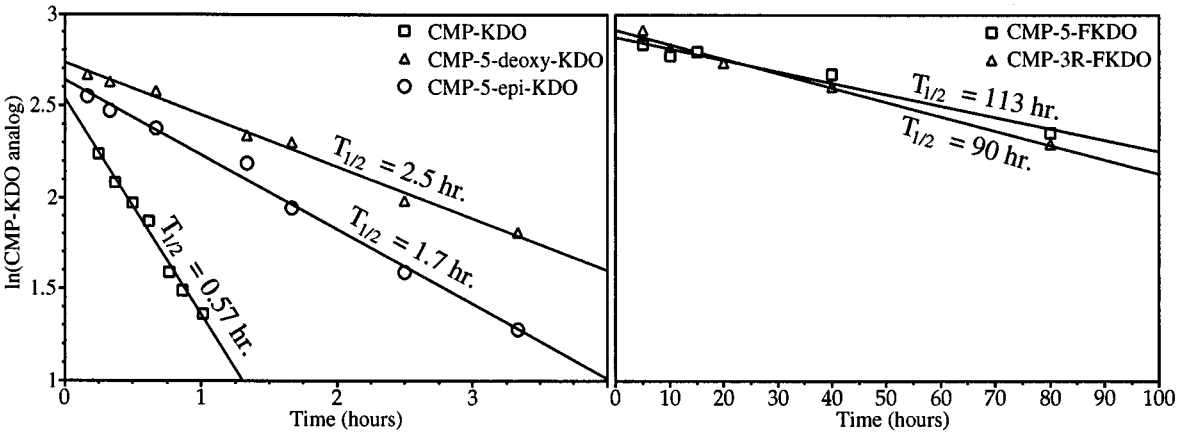


FIGURE 3: Decomposition rates of CMP-KDO and analogs in 20 mM $MgCl_2$ and 100 mM Tris-HCl (pH 7.5). These rates were determined on the basis of the remaining CMP-KDO or analog as measured by NMR analysis.

Table 2: Stabilities of Commonly Found Sugar Nucleotides[a]

nucleotide sugars	half-life (h)
GDP-β-fucose	302[b]
GDP-α-mannose	>168
UDP-α-*N*-acetylglucosamine	>168
UDP-α-galactose	>168
CMP-β-sialic acid	151[c]
CMP-β-KDO	0.57

[a] The study was carried out at 25 °C in 100 mM Tris-HCl (pH 7.5) with 20 mM nucleotide sugar and 20 mM $MgCl_2$. [b] Hydrolysis in a 25 mM phosphate buffer (pH 6.0) containing 10 mM $MnCl_2$ at 20 °C. Without $MnCl_2$ the half-life increased to 3340 h (Murray et al., 1996). The half-life as measured by the NMR method was >168 h. [c] At pH 4, CMP-sialic acid was completely hydrolyzed within 1 h at 37 °C (Dawson et al., 1986).

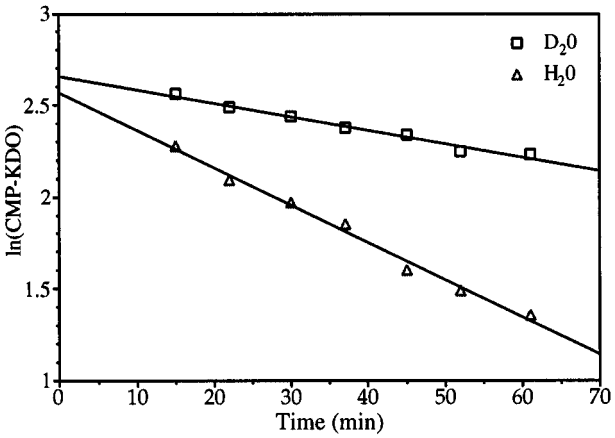


FIGURE 4: Solvent isotope effect on CMP-KDO hydrolysis measured in 20 mM $MgCl_2$ and 100 mM Tris-HCl buffer at 25 °C.

is observed. In the computer dynamics simulations, this hydrogen bond had the effect of preorienting the phosphate group to occasionally accept a hydrogen bond with the hydroxyl at the 4 position, which in turn helped to induce a boat conformation in the six-membered ring structure. Also present were hydrogen-bonding interactions between the phosphate oxygen and the hydroxyl groups at positions 7 and 8. From these results, it is suggested that the hydrogen bond with the hydroxyl functionality at 5 might play a 2-fold role in the hydrolysis of CMP-KDO. In addition to what electron-withdrawing contribution the hydrogen bond may give to the the phosphate, it might also act to reduce the entropic barrier to the hydrolysis of the glycosyl phosphate linkage by preorganizing the phosphate moiety into a

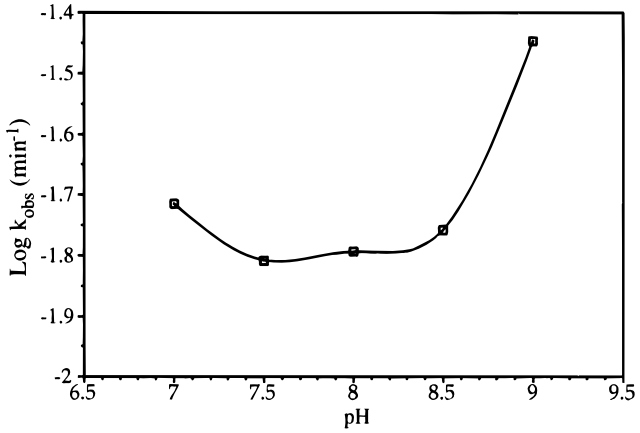


FIGURE 5: pH profile of CMP-KDO hydrolysis measured in 100 mM Tris-HCl and 20 mM $MgCl_2$ at 25 °C.

conformation which is conducive to formation of a boat conformation within the sugar ring with multiple hydrogen-bonding interactions with the phosphate group to facilitate its departure (Scheme 1). The glycosidic cleavage is further encouraged in the presence of magnesium ions, which may complex with the phosphate group.

In summary, this study provides some insights into the mechanism of CMP-KDO hydrolysis. The characteristics of the common mechanism for hydrolysis of glycosides, specific acid A-1 mechanism, are not observed in the hydrolysis of CMP-KDO. Nor can a mechanistic parallel be drawn between CMP-KDO and the related glycosides of sialic acid because of the difference in solvent isotope effects and buffer catalysis. Furthermore, even CMP-5FKDO or CMP-3FKDO with the potent inductive groups are not as stable as CMP-sialic acid. This may be due to the facts that sialic acid has an equatorial 5-acetamido group and a different phosphate conformation with respect to the pyranose ring as well as differences in stereochemistry at the hydroxyls at the 4 and 7 position, which may eliminate what contribution these substituents make to the rate of decomposition of CMP-KDO. These results demonstrate that CMP-KDO hydrolysis is unique. A mechanism with intramolecular H-bonding facilitated by an array of hydroxyl interactions with the phosphate group promotes a twist-boat conformation and possibly a favorable C-1 carboxylic acid conformation. The mechanistic details regarding the timing of proton transfer and the conformational change of the sugar ring during glycosidic cleavage, however, remain to be investi-

gated. Since tremendous efforts have been directed toward the discovery of effective drugs against Gram-negative bacteria, we believe understanding the mechanism of the CMP-KDO hydrolysis will facilitate the development of inhibitors of CMP-KDO synthetase.

ACKNOWLEDGMENT

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