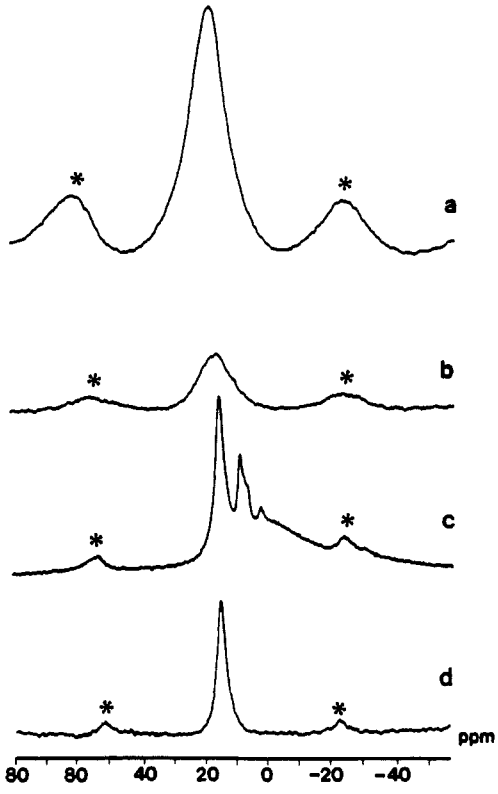

Figure 1. 99.5-MHz proton MAS-NMR spectra. (a) $CaHPO_4$, single-pulse excitation, eight scans, pulse delay 4 s. (b) $CaHPO_4$, obtained by cross polarization from ^{31}P to ^1H ($^1H\{^{31}P\}$ CP), eight scans, pulse delay 60 s. (c) Sample obtained by dissolving KH_2PO_4 in 95% D_2O/5% H_2O followed by evaporation, single-pulse excitation, 100 scans, pulse delay 1 s. (d) Same sample as in c, $^1H\{^{31}P\}$ cross polarization, 12 scans, pulse delay 60 s. * denotes spinning sideband.

applying ^{31}P decoupling during the observation of the ^1H free-induction decay.

Figure 1a is the ^1H MAS spectrum of natural-abundance $CaHPO_4$ obtained with conventional single-pulse excitation. This spectrum shows a single, broad isotropic peak with a chemical shift of 15.3 ppm and a series of spinning sidebands. This spectrum is essentially identical with that of a sample of monetite (natural $CaHPO_4$) reported by Yesinowski and Eckert[3] except that our spectrum is broader due to slower spinning and/or disorder in our sample. Figure 1b is the $^1H\{^{31}P\}$ CP/MAS spectrum of the same sample as in Figure 1a. Sufficiently long pulse delays were used to avoid partial saturation of the proton magnetization in Figure 1a or the phosphorus magnetization in Figure 1b. The signal intensity in Figure 1b is 0.25 that in Figure 1a which compares satisfactorily with the theoretical ratio $\gamma 31_P/\gamma 1_H = 0.40$, demonstrating that $^1H\{^{31}P\}$ cross polarization is reasonably efficient for this sample.

Figure 1c is the ^1H MAS spectrum of a sample that was prepared by dissolving KH_2PO_4 in 95% D_2O/5% H_2O and then evaporating to dryness. The spectrum consists of five isotropic resonances: a broad probe background signal centered at 0 ppm and four signals due to the sample. Drawing on the ^1H MAS-NMR shift assignments for calcium phosphates reported by Yesinowski and Eckert,[3] it is reasonable to propose the following assignments for Figure 1c. The intense peak at 14.0 ppm is assigned to the acid (POH) protons. The peak at 8.0 ppm and shoulder at 5.2 ppm are assigned to two or more types of mobile structural or surface water.[10] Finally, the small peak at 1.1 ppm is consistent with the presence of a small hydroxide impurity, probably as a distinct phase. The $^1H\{^{31}P\}$ CP/MAS spectrum of this sample (Figure 1d) shows a single intense isotropic resonance at 14.0 ppm, a result which is consistent with the above assignments. The other ^1H resonances in Figure 1c (including the probe background signal) are absent from Figure 1d, implying that they are due to protons that are either remote from ^{31}P nuclei or so mobile as to average ^1H-^{31}P dipolar interactions. Identical results were obtained for natural abundance samples of KH_2PO_4 except that the 14.0-ppm signal was broadened by homonuclear dipolar interactions that were incompletely averaged at 4 kHz (spectra not shown). The role of the new experiment in assigning ^1H MAS spectra is reminiscent of that of the interrupted-decoupling experiment[11] (also called dipolar dephasing) in ^{13}C CP/MAS-NMR spectroscopy in that both facilitate spectral assignment in return for a penalty in sensitivity.

It is easy to imagine extensions of this experiment involving, for example, $^1H\{^{13}C\}$ and $^1H\{^{27}Al\}$ CP/MAS experiments on catalyst surface species. In some cases it might be desirable to use the cross polarization process to drain transverse magnetization from protons to their associated low-γ nuclei which, in the case of quadrupolar spins, might have short $T_{1\rho}$ values. Finally, one could imagine using the standard tricks of spin gymnastics to tailor the low-γ magnetization prior to cross polarization or to exploit correlations between two or more nuclei.

Acknowledgment. This work was supported by the National Science Foundation (CHE-8700667) and the Office of Naval Research (N00014-88-K-0239).

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Identification of a Covalent α-D-Glucopyranosyl Enzyme Intermediate Formed on a β-Glucosidase

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The catalytic mechanism of enzymes which hydrolyse glycosides with net retention of anomeric configuration has been a subject of study for many years.[1-4] A double displacement mechanism involving an intermediate of some kind is fairly well-established, but some controversy has existed as to whether this intermediate is covalently bonded or exists as an ion pair and whether the sugar residue involved is cyclic or acyclic.[5] In this paper we describe a series of ^{19}F NMR experiments which identify the intermediate arising from hydrolysis of 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride by a β-glucosidase from *Alcaligenes faecalis* (pABG5 β-glucosidase).[6] In a separate ^{19}F NMR experiment we have proven the α-configuration of the anomeric linkage of this sugar to the enzyme.

A common feature of all the possible mechanisms is that both formation and hydrolysis of the glycosyl enzyme intermediate proceed via transition states with substantial oxocarbonium ion character. This is illustrated in Scheme I for the mechanism involving initial exocyclic bond cleavage and a covalent glucopyranosyl intermediate. We have capitalized on this in our use of 2-deoxy-2-fluoroglycosyl derivatives with good leaving groups, as mechanism-based inactivators,[7,8] since the fluorine at C-2 inductively destabilizes such positively charged transition states, slowing the rates of both glycosyl enzyme formation *and* hy-

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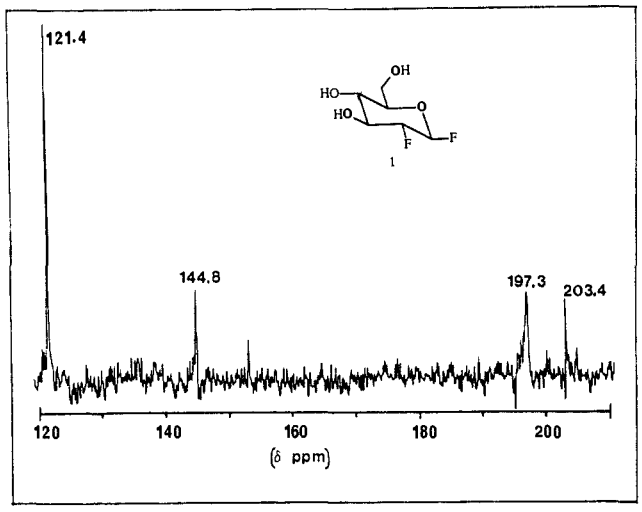
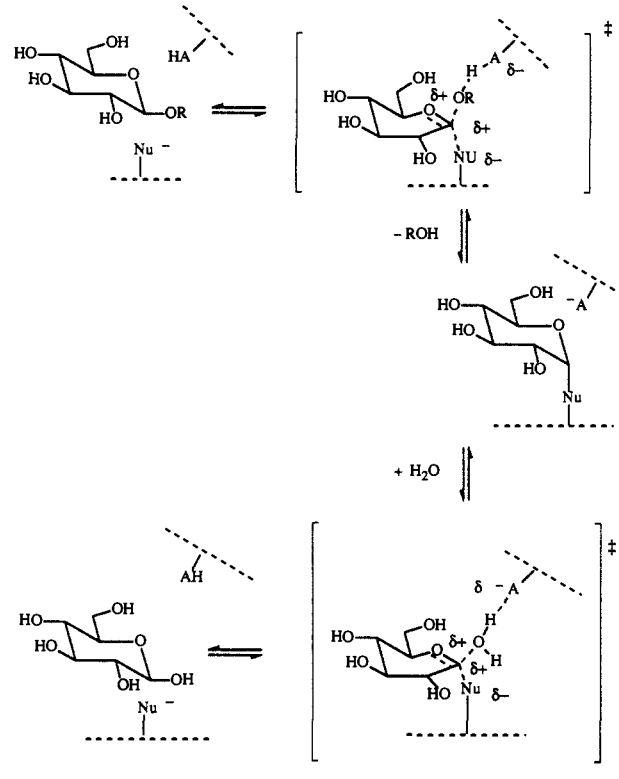


Figure 1. Proton decoupled ^{19}F NMR spectrum of pABG5 β-glucosidase (0.73 mM) and **1** (1.05 mM) in 50 mM sodium phosphate buffer, pH 6.8. This spectrum was recorded using gated proton decoupling (decoupler on during acquisition only) and a 90° pulse angle with a repetition delay of 2s. A spectral width of 40000 Hz was employed and signal accumulated over 10000 transients.

Scheme I



drolysis. The presence of a reactive leaving group increases the rate of glycosyl enzyme formation, resulting in accumulation of the intermediate. Proof of the existence of this intermediate is obtained as follows.

Incubation of pABG5 β-glucosidase with a slight excess of 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (**1**), resulted in rapid inactivation of the enzyme. The ^{19}F NMR spectrum of this sample, shown in Figure 1, clearly indicates that reaction has occurred since the large peak at δ 121.4 ppm is due to released inorganic fluoride. The two small resonances at δ 144.8 and 203.4 ppm are the signals from F-1 and F-2, respectively, of the excess unreacted inhibitor. The remaining broad signal at δ 197.3 ppm must therefore arise from the 2-deoxy-2-fluoro-D-glucopyranosyl enzyme intermediate. The chemical shift observed is consistent with this assignment but provides no clue as to the identity of the enzymic nucleophile since such chemical shifts are relatively

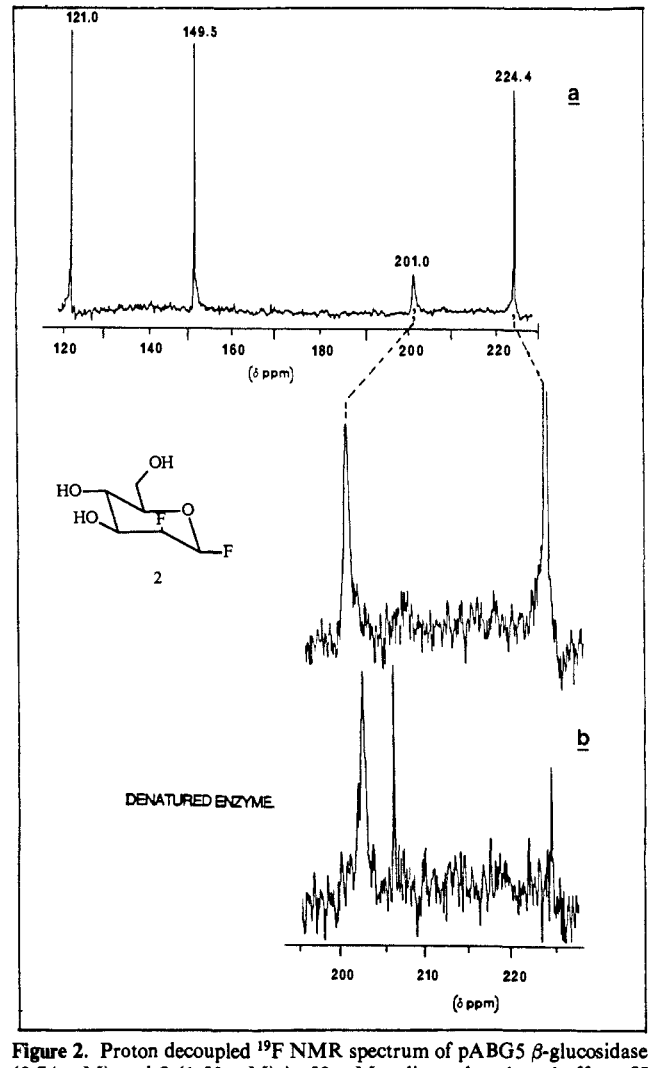


Figure 2. Proton decoupled ^{19}F NMR spectrum of pABG5 β-glucosidase (0.74 mM) and **2** (1.52 mM) in 50 mM sodium phosphate buffer, pH 6.8. Spectra were recorded by using gated proton decoupling (decoupler on during acquisition only) and a 90° pulse angle with a repetition delay of 2s. A spectral width of 40000 Hz was employed, and signal accumulated over 10000 transients for the native protein and 30000 transients for the denatured protein in 8 M urea.

insensitive to the chemical nature of the anomeric substituent. The large line width ($\Delta\nu$ = 130 Hz) is consistent with the fluorine being attached to a macromolecule. Integration of the peaks due to fluoride and the fluoroglucosyl enzyme suggests that the products were indeed formed in approximately equivalent amounts. This was confirmed in a separate experiment involving measurement of the "burst" of fluoride released on reaction with a known amount of enzyme.[9]

The anomeric configuration of this intermediate cannot be deduced simply from the ^{19}F NMR spectrum since the ^{19}F chemical shift of 2-deoxy-2-fluoroglucosides or glycosyl esters is relatively insensitive to anomeric configuration[10] and since any potentially useful coupling constant information is lost in the large natural line width. However, the ^{19}F NMR chemical shifts of 2-deoxy-2-fluoromannosides and -mannosyl esters are very sensitive

(9) Known amounts of pABG5 β-glucosidase were incubated with 2-deoxy-2-fluoro-β-D-glucosyl fluoride, and the amount of fluoride released was determined by the dye binding assay described in the following: Megregian, S. *Colorimetric Determination of Nonmetals, Chemical Analysis*; Boltz, D. F., Howell, J. A., Eds.; Wiley: New York, 1978; Vol. 8, pp 109–137. The β-glucosidase concentration was determined from the extinction coefficient at 280 nm, $E^{0.1\%} = 2.20$ cm^{-1}, which itself was determined by a quantitative amino acid analysis. A stoichiometry of 0.93 (± 0.05) mol of fluoride released per mol of enzyme treated was determined.

(10) Only small differences in ^{19}F chemical shift are generally observed, e.g., the α-anomer of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-D-glucopyranose resonates just some 1.4 ppm upfield from the β-anomer.

to anomeric configuration, with the α-anomers typically resonating some 16–20 ppm downfield from the β-anomers. Fortunately this enzyme also exhibits significant mannosidase activity[11] and is efficiently inactivated by 2-deoxy-2-fluoro-β-D-mannosyl fluoride (**2**).[8] Thus, the 2-deoxy-2-fluoromannosyl enzyme can be formed and its anomeric configuration determined by ^{19}F NMR.

Figure 2a shows the ^{19}F NMR spectrum of pABG5 β-glucosidase (0.74 mM) inactivated with 2-deoxy-2-fluoro-β-D-mannosyl fluoride (1.52 mM). Resonances at δ 121.0, 149.5, and 224.4 ppm arise from released fluoride and F-1 and F-2, respectively, of excess inhibitor. The broad peak at δ 201.0 ppm is the signal from the 2-fluoromannosyl enzyme, a chemical shift which is consistent with an α-anomeric configuration. In order to ensure that the shift observed is not in part due to its local environment,[12] the inactivated 2-deoxy-2-fluoromannosyl enzyme was denatured by overnight dialysis against 8 M urea and the ^{19}F NMR spectrum of this sample determined, Figure 2b. The presence of the peak due to bound inhibitor clearly indicates the covalent nature of the linkage and the fact that only a small shift back upfield ($\Delta\delta$ = 1.6 ppm) is observed indicates that environmental effects were indeed small. Peaks at δ 206.2 and 224.5 ppm arise from α- and β-2-deoxy-2-fluoro-D-mannose which is produced upon nonenzymic hydrolysis of the exposed sugar residue after denaturation.

These experiments have provided the first room temperature spectroscopic evidence, with intact enzyme, for the existence and the α-stereochemistry of this covalent glycopyranosyl intermediate. Such information has only been available previously from indirect trapping methods involving denaturation,[13] derivatization with highly reactive substrate analogues,[3] or to a certain extent by low-temperature trapping.[14]

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Synthesis and Reactivity of Cobalt(III) Complexes Bearing Primary- and Secondary-Side Cyclodextrin Binding Sites.† A Tale of Two CD's

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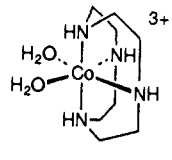
In recent years, efforts to focus the catalytic power of metals by connection to a binding moiety have received considerable attention.[1] Although the cyclen–Co(III) complex (**I**) has exhibited



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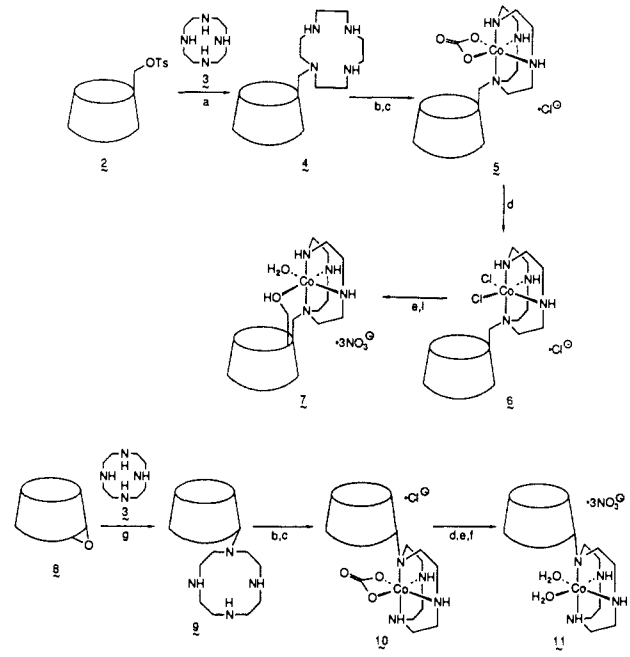
† Dedicated to the memory of Myron L. Bender.

Scheme I[a]



[a] (a) DMF, 90 °C, 24 h; (b) acidification with 1.2 M HCl; (c) $Na_3Co(CO_3)_3 \cdot 3H_2O$, 65 °C, 5 min; (d) MeOH, HCl, 65 °C, 5 min; (e) QAE-Sephadex chromatography (OH^- form); (f) acidification with HNO_3; (g) DMF, 100 °C, 48 h.

amongst the greatest rate accelerations in acyl-[2] and phosphoryl-[3,4]transfer reactions, this catalytic unit has not been used previously in the design of a preassociating artificial metalloenzyme. Cyclodextrin (CD) has been used previously for the synthesis of metalloprotein models,[1a,b,j,5] but incorporation of a Co(III) metal center onto CD has not been achieved to date. Accordingly, we have synthesized two cyclen–Co(III) complexes positioned alternately on the primary- and secondary-sides of β-CD. We now report that artificial metalloenzyme **7** demonstrates a 900-fold enhancement of metal-promoted ester hydrolysis attributable entirely to the presence of the CD binding unit. Furthermore, we find that the properties of primary- and secondary-side CD's are unexpectedly quite different, in part because the primary-side derivative is able to involve an adjacent hydroxymethyl group in chelation of the metal ion.

The syntheses were accomplished as shown in Scheme I. Reaction of cyclen (**3**) with β-CD monotosylate (**2**)[6] afforded, after

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