Hydration of Cellobial by Exo- and Endo-Type Cellulases: Evidence for Catalytic Flexibility of Glycosylases†

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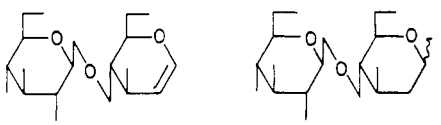
ABSTRACT: New insight has been obtained into the catalytic capabilities of cellulase. Essentially homogeneous preparations of exo- (or Avicelase-) type and endo- (or CMCase-) type cellulases from *Irpex lacteus* and *Aspergillus niger*, respectively, were shown to hydrate the enolic bond of cellobial to form 2-deoxycellobiose. The *A. niger* enzyme also synthesized a small amount of a 2-deoxycellobiosyl-transfer product from cellobial. By use of digests conducted in deuterated buffer and ^{1}H NMR spectra for product analysis, both cellulases were found to protonate (deuterate) the double bond of cellobial from below the *si* face of the D-glucal moiety, i.e., from a direction opposite that assumed for protonation of the β-D-glycosidic linkages of cellulose and cellodextrins. The exo enzyme, which hydrolyzes the latter substrates primarily to cellobiose, rapidly catalyzed cellobial hydration to produce the β-anomer of β-D-glucopyranosyl(1→4)-2-deoxy-D-glucose-2(*e*)-*d*. The *A. niger* cellulase produced the same 2-deoxycellobiose-*d* from cellobial, though too slowly for its configuration to be determined. However, evidence was obtained for the formation of a β-2-deoxycellobiosyl-*d*-D-glucose-transfer product by the enzyme. Thus, it is likely that all of the observed reactions with cellobial represent trans additions at the double bond. In any case, the anomeric configuration of products is created de novo. Separate mechanisms are described for the reaction of cellobial hydration and for the stereochemically different reaction of cellulose hydrolysis catalyzed by the present enzymes, assuming an arrangement of their catalytic groups analogous to that found in lysozyme. The results with the cellulases add to growing evidence for the view that glycosylases have catalytic groups that are functionally flexible beyond the needs of the principle of microscopic reversibility and, hence, the potential to act upon different substrates by different mechanisms.

Cellulases have traditionally been considered to act productively only on β-O-glycosidic linkages, specifically of cellulosic materials, cellodextrins, aryl β-cellobiosides, etc. However, as reported in abstract form (Kanda et al., 1981), we have obtained evidence of the ability of highly purified cellulases of two different types to catalyze hydration of the double bond of cellobial (I) to form 2-deoxycellobiose (II).



I II

These experiments were undertaken as an extension of studies on glycosylation reaction catalyzed without glycosidic bond cleavage, as such studies had provided new insights into the capabilities and mechanisms of α-amylases (Hehre et al., 1971, 1973; Okada et al., 1979), β-galactosidase (Lehmann & Schröter, 1972; Lehmann & Zeiger, 1977; Brockhaus & Lehmann, 1977), α- and β-glucosidases (Hehre et al., 1977, 1980), β-amylase (Hehre et al., 1973, 1979, 1981), and glucoamylase and glucodextranase (Kitahata et al., 1981).

This paper describes the nature and steric course of reactions with cellobial catalyzed by the exocellulase of *Irpex lacteus* and the endocellulase of *Aspergillus niger* and examines the relationship between cellobial hydration and cellulose hydrolysis catalyzed by these enzymes. The findings that both cellulases protonate cellobial from a direction opposite that generally assumed for β-glycosidic substrates and convert this prochiral substrate to products of β-configuration reveal a stereochemical course for cellobial hydration that differs from the stereochemical course (involving retention of configuration) found for cellodextrin hydrolysis by the same *I. lacteus* and *A. niger* enzymes (Kanda et al., 1978; Okada, 1985). Separate mechanisms are presented for the reactions of cellobial hydration and cellulose hydrolysis effected by the cellulases, involving an arrangement of catalytic groups at the active site able to support the two mechanisms. The results are discussed in terms of related findings and of the concept that carbohydrases are glycosylases with functionally flexible catalytic groups that provide both the ability to create product configuration de novo and the ability to catalyze reactions with different substrates by different mechanisms.

Experimental Procedures

General Methods. Paper chromatography (decending) was carried out with 1-butanol/ethanol/water (13:8:4) and 23 × 56 cm sheets of Whatman No. 1 or prewashed 3MM paper. Staining was by a silver nitrate dipping method (Trevelyan et al., 1950) with papers hung in air for 12 min following treatment with 2% sodium hydroxide in ethanol. Thin-layer chromatography was performed with chloroform/ether (2:1)

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and silica gel G plates; spots were visualized by the sulfuric acid–char method. Concentrations were effected with rotary vacuum evaporators at 30 °C unless otherwise noted; drying was done in a vacuum oven at or below 37 °C. Crystallinity and birefringence were judged by use of a polarization microscope equipped with a second-order (red) retardation plate. Melting points were determined on a Mel-Temp block (Laboratory Devices, Cambridge, MA) and are uncorrected. Optical rotations were measured on a Rudolph and Sons Model 70 polarimeter with 2-dm tubes. Total sugar was determined by the phenol–sulfuric acid method (Dubois et al., 1956) standardized with glucose, 2-deoxyglucose, or an equimolar mixture of the two, as appropriate. Free 2-deoxycellobiose was determined by a micromodification of the method of Park and Johnson (1949) standardized with a sample of the pure sugar; measurements were made in triplicate. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

Proton NMR spectra were recorded at 200 or 220 MHz on Varian spectrometers operated in the Fourier-transform mode. Spectra were recorded in dueterium oxide (99.8 atom % D, Stoehler Isotope Chemicals, Waltham, MA) or in acetate-d_4/deuterium oxide buffers at ambient temperature. Chemical shift measurements refer to 3-(trimethylsilyl)propanesulfonic acid sodium salt.

Cellulase Preparations. Cellulase Ex-1 from the wood-rotting fungus *Irpex lacteus* (*Polyporus tulipferae*) had been purified to essential homogeneity and gave a single band on SDS[1]–polyacrylamide electrophoresis (Kanda et al., 1978). It readily hydrolyzed insoluble cellulosic materials such as filter paper, cotton yarn, or Avicel (a microcrystalline cellulose powder). Its high saccharifying activity for Avicel, 0.057 μmol of reducing sugar (calculated as glucose) min^{-1} mg^{-1}, was in contrast to its very low saccharifying activity (0.001 μmol min^{-1} mg^{-1}) with CMC (sodium carboxymethylcellulose of degree of substitution 0.63).[2] Cellohexaose and cellopentaose were rapidly hydrolyzed compared to the tetraose or triose; cellobiose and aryl β-D-glucosides were not attacked. Ex-1, considered to be an exo- or Avicelase-type cellulase that acts with retention of configuration (Kanda et al., 1978), catalyzed the nearly exclusive production of cellobiose from cellulose and cellodextrins although some glucose was also formed from cellotriose and cellopentaose. Ex-1, however, is not a strict cellobiosyl mobilizing enzyme (β-1,4-glucan cellobiohydrolase) as it has been found to catalyze glucosyl- as well as cellobiosyl-transfer reactions from cellotriose, cellopentaose, and *p*-nitrophenyl β-cellobioside (Kanda et al., 1983).

The cellulase from *Aspergillus niger*, a typical endo-type enzyme, was purified to essential homogeneity by a multistep procedure. It gave a single protein band on polyacrylamide gel as well as on ampholine electrophoresis (Okada, 1985). The enzyme had very slight activity with Avicel or other water-insoluble forms of cellulose, but it had a high specific activity [50.5 μmol of reducing sugar (calculated as glucose) min^{-1} mg^{-1}] with CMC. Cellohexaose was rapidly hydrolyzed to form the di-, tri-, and tetraoses, as well as a little glucose. The products of cellopentaose hydrolysis showed upward mutarotation, indicating that the enzymic reaction proceeded with retention of configuration (Okada, 1985). Cellotriose was only slowly hydrolyzed; cellobiose and aryl β-D-glucosides were not attacked.

Hepta-O-acetyl-α-cellobiosyl Bromide. Preparation was by Fisher and Zemplen's (1910) method: 20 g of crystalline α-cellobiose octaacetate, $[\alpha]^{18}_D$ +41.8°, was dissolved in 80 mL of chloroform and treated with 75 mL of 30% hydrogen bromide in acetic acid (Eastman-Kodak). After 45 min at 25 °C, the solution was shaken with 600 mL of ice–water. The separated chloroform phase was washed twice with 300 mL of ice–water and dried with sodium sulfate. Treatment with 2 volumes of ethyl ether yielded a crystalline precipitate that was recovered on a sintered glass filter and dried in a vacuum oven; the product (17.5 g) gave a single spot on TLC (chloroform/ether, 2:1). It was recrystallized by treating a solution in chloroform with an equal volume of ether; storage at 4 °C overnight gave highly birefringent crystals of pure hepta-*O*-acetyl-α-cellobiosyl bromide: yield 15.0 g; $[\alpha]^{22}_D$ +95.1° (*c* 2, chloroform); mp 191–192 °C (discolored at 179–184 °C) [lit. (Fisher + Zemplen, 1910) $[\alpha]^{20}_D$ +95.3° (chloroform), discolored at 180 °C with mp a few degrees higher and variable; (Brauns, 1926) $[\alpha]^{22}_D$ +95.8° (chloroform)].

Hexa-O-acetylcellobial. Recrystallized α-cellobiosyl bromide heptaacetate, 4.5 g, was dissolved in 75 mL of cold 90% acetic acid containing 0.25 mg of chloroplatinic acid (Bergmann & Schotte, 1921). After addition of 30 g of pulverized zinc, the mixture was vigorously stirred for 4 h at 10 °C and then passed through a sintered glass filter. The clear filtrate was poured into 800 mL of ice–water and kept at 4 °C overnight. The granular precipitate was recovered, brought to near dryness in a vacuum oven, and then dissolved in 15 mL of chloroform. On treatment with 45 mL of petroleum ether, large birefringent crystals of cellobial hexaacetate were formed that were dried in a vacuum oven at 34 °C. The product (1.84 g) gave a single spot on TLC and had $[\alpha]^{21}_D$ −20.8° (*c* 1, chloroform) and mp 122–124 °C [lit. (Fisher & von Fodor, 1914) $[\alpha]^{20}_D$ −19.8° (tetrachloroethane), mp 134–135 °C; (Haworth et al., 1930) $[\alpha]^{18}_D$ −20° (chloroform), mp 137 °C].

Cellobial. Hexa-*O*-acetylcellobial, 1.75 g, was deacetylated with 25 mL of 0.01 M sodium methoxide in dry methanol (25 °C, 20 h). After solvent removal under vacuum at 30 °C, the product was dissolved in 30 mL of warm 95% ethanol, kept overnight at 4 °C, and recovered as sturdy birefringent crystals. The pure compound gave a single spot on chromatograms (R_{Glc} 1.52) and had $[\alpha]^{21}_D$ +0.9° (*c* 0.7, water), mp 181–183 °C (discolored at 173 °C), and a reducing power [Park and Johnson (1949) method] equivalent to only $^1/_{200}$ of that of 2-deoxycellobiose [lit. (Fisher & von Fodor, 1914) $[\alpha]_D$ +1.0° (water), mp 175–176 °C; (Haworth et al., 1930) $[\alpha]^{17}_D$ +1° (water), mp 171 °C]. Bromine addition at the double bond was 94–95% of the theoretical. ^{1}H NMR data are described under Results. Anal. Calcd for $C_{12}O_9H_{20}$: C, 46.75; O, 46.71; H, 6.54. Found: C, 46.50; O, 46.56; H, 6.47. When used as a substrate in enzymic digests carried out in deuterium oxide, the required amount of cellobial was dissolved in methyl alcohol-*d* (99 atom % D, C.E.A. Service Molecules Marquees, Gif sur Yvette, France) and dried under vacuum at 30 °C just before addition of enzyme.

2-Deoxycellobiose [β-D-Glucopyranosyl(1→4)-2-deoxy-D-glucose] Standard. Crystalline cellobial (320 mg) was dissolved in 5 mL of cold 0.2 N sulfuric acid, held 48 h at 4 °C, and then chromatographed on sheets of 3MM paper. Silver nitrate stained guide strips showed much unchanged cellobial (R_{Glc} 1.5), accompanied by material migrating at R_{Glc} 0.7–1.1,

[1] Abbreviations: SDS, sodium dodecyl sulfate; CMC, carboxymethylcellulose.

[2] The statement on p 1221 of Kanda et al. (1978) that "The ratio of Ex-1 activity for Avicel to that for CMC was about 9.6..." might appear to contradict this. However, this low ratio refers to a comparison made of reducing sugar formed per milliliter by a digest of Avicel with 0.3 mg/mL enzyme vs. a digest of CMC with 3.6 mg/mL enzyme.

which proved to be a mixture of glucose and 2-deoxycellobiose. On rechromatography of the mixture on No. 1 paper, 45.2 mg of pure 2-deoxycellobiose (R_{Glc} 0.84) was recovered as a white amorphous powder, $[\alpha]^{23}_D$ +27.8° (*c* 0.5, water) [lit. (Bergmann & Breuers, 1929) [α] +23.2° (water)]. ^{1}H NMR data are given under Results. The product was used as a reference standard for paper chromatography and for the Park and Johnson (1949) reducing sugar method, allowing determinations of 2-deoxycellobiose to be made in enzyme/cellobial reaction mixtures.

Deuterated Products Recovered from Enzyme/Cellobial Digests. I. lacteus Ex-1 cellulase, 3.9 mg in 0.60 mL of 0.05 M acetate-d_4/deuterium oxide buffer of pD 4.9 (99.8 atom % D), was added to 60 μmol of cellobial freshly dried from solution in methyl alcohol-*d*. (The enzyme had been dialyzed for 24 h at 8 °C against the pD 4.9 buffer to exchange replaceable ^{1}H for ^{2}H atoms.) Following direct ^{1}H NMR examination of the digest during 2 h at 20 °C, it was incubated an additional 3 h at 25 °C then chromatographed along with 2-deoxycellobiose end markers on sheets of Whatman No. 1 paper. Material migrating at the marker position (R_{Glc} 0.84) was eluted with methanol; its yield (phenol–sulfuric acid method) was 29.8 μmol, as 2-deoxycellobiose. A sample (20 μmol) was treated with 24 units of purified sweet almond β-glucosidase (Boehringer) in 0.6 mL of pH 5.4 acetate buffer. After incubation (30 °C, 6.5 h), the mixture was chromatographed (22 h) no No. 1 paper, and the reaction products were eluted; 17.4 μmol of 2-deoxy-D-glucose-*d* (R_{Glc} 1.87) and 16.7 μmol of glucose were found (phenol–sulfuric acid method). A proton NMR spectrum of a 25 mM solution of the recovered 2-deoxy-D-glucose-*d* in D_2O showed resonance doublets at 5.4 and 4.9 ppm, due to the anomeric protons of α- and β-2-deoxy-D-glucose, and also multiplets at 1.75 and 1.5 ppm assignable to the axial C-2 protons of α- and β-2-deoxy-D-glucose (Hall & Manville, 1968; Hehre et al, 1977). No resonance at 2.1 or 2.3 ppm, referable to an equatorial C-2 proton of either the α- or β-form of the sugar, was present.

A. niger cellulase, 4.5 mg in 1.0 mL of 0.1 M acetate-d_4/deuterium oxide buffer of pD 5.3 (previously dialyzed against the buffer at 8 °C during 24 to exchange ^{1}H for ^{2}H atoms), was added to 100 μmol of cellobial (freshly dried from solution in methyl alcohol-*d*). The mixture was incubated at 30 °C for 10 h and then chromatographed on two sheets of prewashed 3MM paper along with glucose as marker at the ends. After the end strips were stained, material at R_{Glc} 0.84 was eluted with methanol from the unstained panels. The yield of the recovered hydration product, calculated as 2-deoxycellobiose, was found to be 14 μmol by the phenol–sulfuric acid method. Following rechromatography on No. 1 paper to remove minor impurities, the product was dried under vacuum from 1.0 mL of deuterium oxide, redissolved in 0.60 mL of deuterium oxide, and subjected to NMR spectroscopic examination (see Results).

A second reaction product, evident as a weakly stained spot at R_{Glc} 0.65 in chromatograms of *A. niger*/cellobial digests, was separated for study. Recrystallized cellobial (100 μmol, dried in vacuo from deuterated methanol) was treated with 10 mg of *A. niger* cellulase in 1 mL of 0.02 M acetate-d_4/D_2O buffer of pD 5.3; the enzyme had been exhaustively dialyzed against the buffer. The digest was incubated at 30 °C for 7.5 h and then chromatographed on sheets of washed 3MM paper along with glucose end markers. The R_{Glc} 0.65 product was eluted with a methanol/water mixture (3:2 v/v). The product was purified by rechromatography on No. 1 paper, then dissolved in 1 mL of deuterium oxide, passed through a Swinnex 0.45-μm HA filter, and dried under vacuum. The product was finally redissolved in 0.6 mL of deuterium oxide (99.8 atom % D) and examined by ^{1}H NMR spectroscopy (see Results). A sample was digested (30 °C, 5 days) with purified sweet almond β-glucosidase (50 units/mL) and then chromatographed to recover the digestion products. Stained guide strips showed spots corresponding to glucose and to 2-deoxy-D-glucose (R_{Glc} 1.87), the former larger. Each eluted product was filtered through a Swinnex 0.45-μm membrane and analyzed by the phenol–sulfuric acid method.

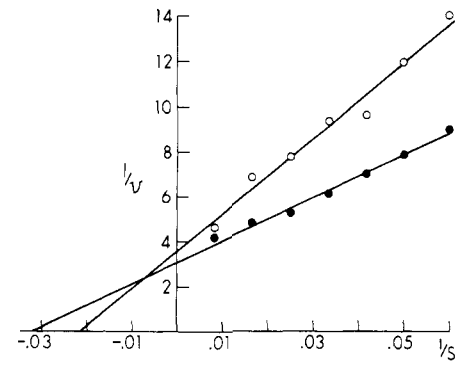


FIGURE 1: Rates of cellobial hydration catalyzed by 0.1 mg/mL *Irpex lacteus* exocellulase in digests (30 °C, 200 min): (●) in 0.1 M pD 4.9 acetate-*d*/D_2O buffer; (○) in 0.1 M pH 4.9 acetate buffer, $1/v$ is in units of (μmol of 2-deoxycellobiose formed enzymically min^{-1} mg^{-1})$^{-1}$; $1/S$ is in units of μmol of cellobial/mL)$^{-1}$.

RESULTS

An exocellulase (Ex-1) from *Irpex lacteus* and an endocellulase from *Aspergillus niger*, both purified to essential homogeneity, were found to utilize cellobial [β-D-glucopyranosyl(1→4)-D-glucal] as a glycosyl substrate by attacking its enolic bond. In initial tests, mixtures of 40 mM cellobial plus 0.2 or 4 mg/mL Ex-1 cellulase, 10 mg/mL *A. niger* cellulase, or pH 4.9 buffer alone were incubated (30 °C, 5 h) and examined by paper chromatography. Both Ex-1 digests differed from the cellobial/buffer control in showing a prominent spot (R_{Glc} 0.84) corresponding to the 2-deoxycellobiose marker; the digest with 4 mg/mL enzyme showed little remaining cellobial substrate. The R_{Glc} 0.84 product, eluted and incubated with β-glucosidase, yielded glucose and 2-deoxy-D-glucose (recovered by chromatography) in a 0.9:1.0 molar ratio. The *A. niger* endocellulase digest chromatogram showed a modest spot corresponding to 2-deoxycellobiose, plus a second (very weakly stained) spot at R_{Glc} 0.65. In other tests, the Park and Johnson (1949) method was used to measure the 2-deoxycellobiose formed upon the hydration of 40 mM cellobial by 0.1 mg/mL Ex-1 or 1.1 mg/mL *A. niger* cellulase in digests (30 °C, 4 h) buffered at pH 4.9. Initial hydration rates were found to be 0.23 μmol of 2-deoxycellobiose formed min^{-1} mg^{-1} for Ex-1 cellulase and 0.027 μmol min^{-1} mg^{-1} for the *A. niger* cellulase, corrected for 1.2% hydration found in a concurrently incubated cellobial/buffer control.

The substantial speed with which the *I. lacteus* exocellulase attacked 40 mM cellobial suggested that the steric course of the catalyzed hydration reaction might be determined by ^{1}H NMR spectroscopy, provided that the enzymic reaction in D_2O also proceeded at a good rate. This proved to be the case. Figure 1 illustrates Lineweaver–Burk plots comparing the initial rates of 2-deoxycellobiose formation from 5–120 mM cellobial by Ex-1 cellulase, catalyzed in aqueous buffer of pH 4.9 and in deuterated buffer of pD 4.9. Least-squares calculations showed that at pH 4.9 K_m = 31.2 ± 3.1 mM cellobial and V_{max} = 0.324 ± 0.014 μmol min^{-1} mg^{-1} and at pD 4.9 K_m

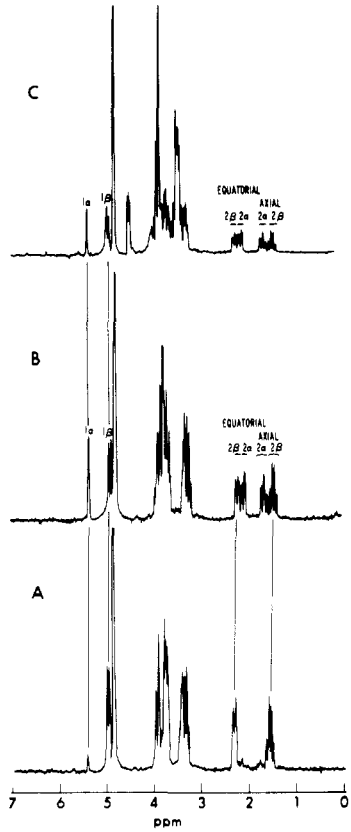


FIGURE 2: ^{1}H NMR reference spectra, recorded in D_2O at 220 MHz, illustrating assignment of the H-1, H-2(e), and H-2(a) resonances of α- and β-2-deoxycellobiose. (A) Freshly dissolved (5 min, 20 °C) 2-deoxy-β-D-glucose, 100 mM; (B) same solution as in (A) after 2 h at 20 °C; (C) equilibrated 2-deoxycellobiose (acid hydration product of cellobial), 48 mM.

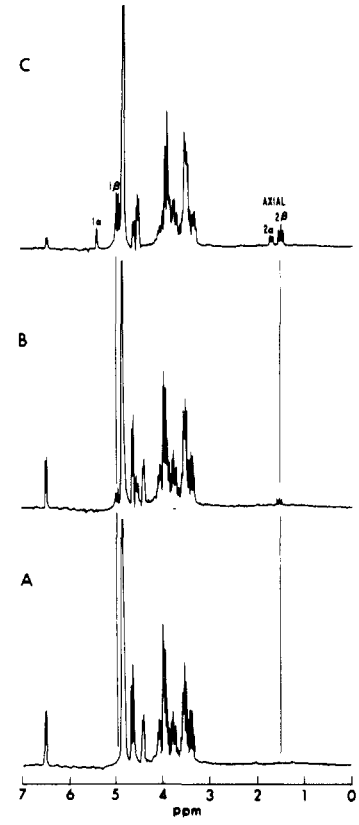


FIGURE 3: ^{1}H NMR spectra at 220 MHz of a digest of 100 mM cellobial and 6.5 mg/mL Ex-1 cellulase in 0.1 M acetate-d_4/D_2O buffer, pD 4.9, incubated at 20 °C: (A) 3–5, (B) 25–28, and (C) 120 min.

= 46.5 ± 8.1 mM cellobial and V_{max} = 0.280 ± 0.021 μmol min^{-1} mg^{-1}.

In preparation for ^{1}H NMR spectroscopic examination of the stereochemistry of the enzymic reactions catalyzed with cellobial, reference spectra at 220 MHz were recorded (in D_2O) of 2-deoxy-β-D-glucose, anomerized 2-deoxy-D-glucose, and anomerized 2-deoxycellobiose prepared by the acid-catalyzed hydration of cellobial. The aim was to identify the H-1, H-2(e), and H-2(a) resonances of the 2-deoxy-D-glucose moiety of α- and β-2-deoxycellobiose. Unequivocal assignments were achieved since the chemical shift and form of each resonance was found to correspond exactly to an assigned resonance of free α- or β-2-deoxy-D-glucose (Figure 2). Thus, the H-1 resonance at 4.93 ppm ($J_{1,2}$ = 9.7 Hz) in the spectrum of freshly dissolved 2-deoxy-β-D-glucose (Figure 2A) and the multiplets at 2.3 and 1.5 ppm due respectively to the H-2(e) and H-2(a) protons of the β-sugar have exact counterparts in the spectrum of anomerized 2-deoxycellobiose (Figure 2C). Likewise, the prominent H-1 resonance at 5.38 ppm ($J_{1,2}$ = 3.1 Hz) of 2-deoxy-α-D-glucose in the spectrum of the anomerized sugar (Figure 2B) and the multiplets at 2.1 and 1.7 ppm due respectively to the H-2(e) and H-2(a) protons of the α-sugar are identifiable in the 2-deoxycellobiose spectrum (Figure 2C). The resonance at 4.52 ppm ($J_{1,2}$ = 7.9 Hz) (Figure 2C) is assigned to the anomeric proton of the β-D-glucosyl residue of 2-deoxycellobiose.

Figure 3 illustrates ^{1}H NMR spectra recorded in the course of a reaction catalyzed by *I. lacteus* Ex-1 cellulase (6.5 mg/mL) with 100 mM cellobial in acetate-d_4/D_2O buffer of pD 4.9; both enzyme and substrate had their ^{1}H atoms exchanged for ^{2}H atoms prior to mixing. The initial spectrum (A), recorded after 3–5 min at 20 °C, shows essentially only the proton resonances of cellobial, i.e., H-1 at 6.48 ppm ($J_{1,2}$ = 5.7 Hz), H′-1 (anomeric proton of the β-D-glucosyl moiety) at 4.61 ppm ($J_{1,2}$ = 7.9 Hz), and H-3 at 4.36 ppm. After a 25–27-min incubation at 20 °C, three new resonances are seen (Figure 3B), that is, a doublet at 4.95 ppm ($J_{1,2}$ ca. 10 Hz) due to the anomeric proton (1_β) of β-2-deoxycellobiose, a doublet at 4.51 ppm ($J_{1,2}$ = 7.9 Hz) assignable to H′-1, the anomeric proton of the β-D-glucosyl residue of 2-deoxycellobiose, and a multiplet at 1.5 ppm due to the H-2(a) proton of β-2-deoxycellobiose. Spectrum C, recorded after 2 h at 20 °C, shows partial (nonenzymic) anomerization of the β-2-deoxycellobiose enzymic product. The absence of resonance signals at 2.1 and 2.3 ppm, for the C-2(e) proton of α- and β-2-deoxycellobioses, respectively, reveals that a deuteron is present at the equatorial position at C-2.

The Ex-1 cellulase thus catalyzes the transhydration of cellobial, protonating (deuterating) the double bond from below and producing the β-anomer of 2-deoxycellobiose. Examination of the reaction product, separated by chromatography of the digest following the above NMR spectroscopy, confirmed its structure. The isolated product (R_{Glc} 0.84) was hydrolyzed by sweet almond β-glucosidase to produce equimolar glucose and 2-deoxy-D-glucose-*d*. A ^{1}H NMR spectrum of the latter, recorded in D_2O, showed it to be 2-deoxy-D-glucose-*2(e)-d*.

The rate of utilization of cellobial by *A. niger* cellulase was too low to permit determining the steric course of the reaction(s) by the ^{1}H NMR approach used with the Ex-1 enzyme. Information on the stereochemistry was, however, obtained by an alternate approach. A digest of 100 μmol of cellobial and 4.5 mg of *A. niger* cellulase in 1 mL of 0.1 M acetate-d_4/D_2O buffer (pD 5.3) was incubated at 30 °C for 10 h and

then chromatographed; the product migrating at R_{Glc} 0.84 (14 μmol calculated as 2-deoxycellobioase) was recovered. A ^1H NMR spectrum, in D_2O, was indistinguishable from that illustrated in Figure 3C for the anomerized 2-deoxycellobiose-2(*e*)-*d*, which ultimately appears in digests of cellobial with Ex-1 cellulase. Cellobial hydration by the *A. niger* enzyme, as by the Ex-1 enzyme, thus involves protonation of the substrate's double bond from below the ring.

Further information on cellobial utilization by *A. niger* endocellulase was obtained by separating and examining the minor (R_{Glc} 0.65) product from 1 mL of a digest (30 °C, 7.5 h) containing 100 μmol of cellobial and 10 mg/mL enzyme in 0.02 M acetate-d_4/D_2O buffer of pD 5.3. A ^1H NMR spectrum of the separated product, recorded in D_2O at 200 MHz and 20 °C, revealed a prominent multiplet at 1.5 ppm and a doublet at 4.93 ppm ($J_{1,2}$ = 9.7 Hz) due respectively to the H-2(a) and H-1(a) protons of a 2-deoxy-β-D-glucosyl moiety. These findings, coupled with the complete absence of H-2 and H-1 resonances of 2-deoxy-α-D-glucose and the absence of a resonance at 2.3 ppm of the H-2(e) proton of 2-deoxy-β-D-glucose, show the R_{Glc} 0.65 product to contain a glycosidically linked 2-deoxy-β-D-glucosyl-2(*e*)-*d* residue. (An incubated control of 100 μmol of cellobial in 1 mL of the pD 5.3 buffer was chromatographed; ^1H NMR spectra of an extract of the R_{Glc} 0.65 region showed no 2-deoxy-D-glucose resonance.) The data clearly show that the formation of the product involves cellobial protonation (deuteration) by the endocellulase from below the double bond and that the reaction represents a trans addition at the double bond. In other analyses, the R_{Glc} 0.65 product was found to be hydrolyzed by sweet almond β-glucosidase to glucose and 2-deoxyglucose, recovered chromatographically in a molar ratio of 1.74:1.0. Together, the findings indicate that the slowly migrating minor product of the action of *A. niger* cellulase on cellobial is a trisaccharide, β-D-glucosyl(1→4)-2-deoxy-β-D-glucosyl-2(*e*)-*d*-(1→?)-D-glucose, presumably formed by β-2-deoxycellobiosyl transfer to the C-4 carbinol of glucose.

Discussion

New insight has been obtained into the catalytic capabilities of cellulase. Essentially homogeneous preparations of a "less random" (exo- or Avicelase-type) and "more random" (endo- or CMCase-type) cellulase, from *I. lacteus* and *A. niger*, respectively, were shown to hydrate the enolic bond of cellobial to form 2-deoxycellobiose. The *A. niger* cellulase in addition synthesized a small amount of a 2-deoxycellobiosyl-transfer product when incubated with cellobial.

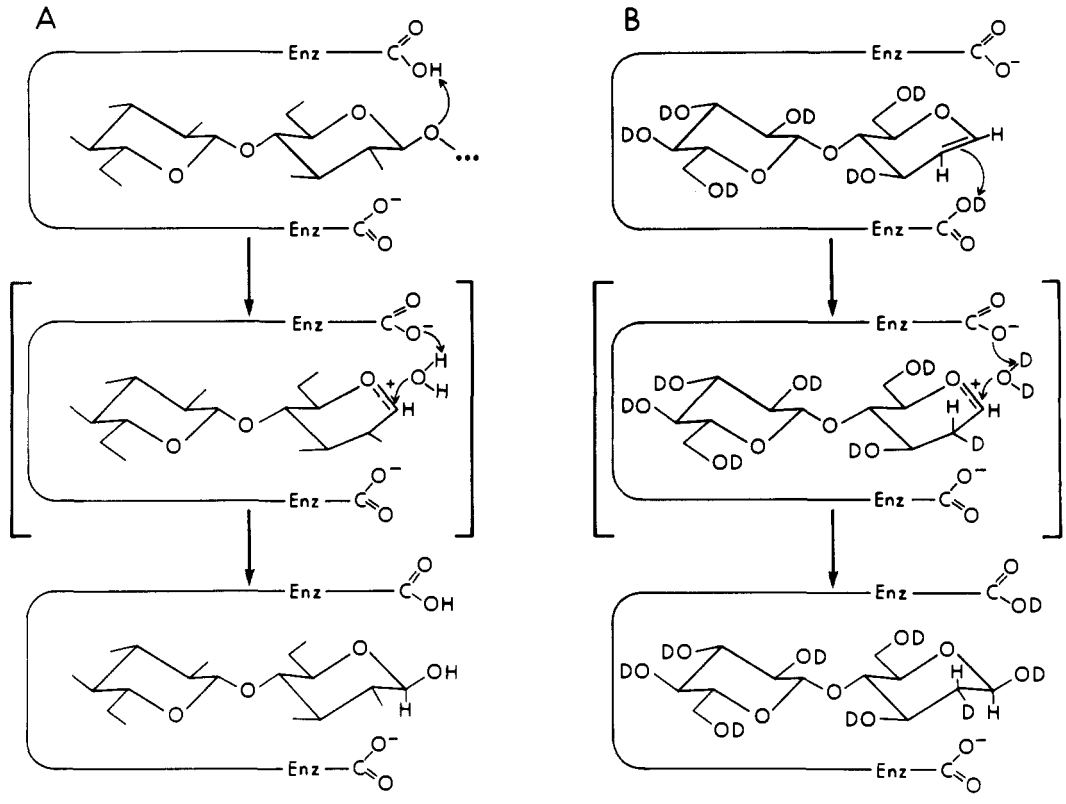
By conduction of the enzymic reactions in deuterated buffer and use of ^1H NMR spectra to characterize the products, clear evidence was obtained that in each case the cellulase protonated (deuterated) cellobial from below the *si* face of the D-glucal residue, i.e., from a direction opposite that assumed for protonation of the β-D-glycosidic linkages of conventional substrates. Also, with the exocellulase from *I. lacteus*, the catalysis of cellobial hydration in D_2O was sufficiently rapid (V_{max} = 0.28 μmol min^{-1} mg^{-1}) to allow the β-anomeric configuration of the 2-deoxycellobiose product to be demonstrated by following the reaction with ^1H NMR spectra. Although the *A. niger* enzyme catalyzed the hydration reaction too slowly to allow the determination of product configuration, clear evidence was obtained for the presence of a cellobial-derived β-2-deoxycellobiosyl residue (glycosidically linked to D-glucose) in the enzymically synthesized transfer product. Thus, it appears highly probable that each of the cellulase-catalyzed reactions observed with cellobial represents a trans addition to the double bond of the substrate. Finally, both cellulases are seen to be capable of creating product configuration de novo, specifically controlling the anomeric form without reference to donor substrate configuration. This capability, first demonstrated with α- and β-glucosidases acting on D-glucal (Hehre et al., 1977) and β-galactosidase acting on D-galactal (Lehmann & Zeiger, 1977), has also recently been shown for several other glycosylases long believed restricted to catalyzing reactions that always invert (or always retain) substrate configuration (Hehre et al., 1980, 1981; M. Gäbelein et al., unpublished experiments; Schlesselmann et al., 1982; Klein et al., 1982).

We believe that the reactions catalyzed with cellobial by the present cellulase preparations are, in each case, due to cellulase itself. Both enzymes were purified to essential homogeneity and had extremely high cellulolytic activity, selective for either microcrystalline cellulose (Ex-1) or for carboxymethylcellulose (*A. niger* endocellulase). In the case of Ex-1, the nearly exclusive production of cellobiose from insoluble cellulose and cellooligosaccharides (Kanda et al., 1978, 1983) shows the enzyme to have a high affinity for binding a terminal cellobiosyl residue (and doubtless cellobial) at the catalytic center. The isolation of exocellulases ("β-1,4-glucan cellobiohydrolases") having similar characteristics from sources other than *I. lacteus* (Wood & McCrae, 1972, 1977; (Berghem & Pettersson, 1973; Halliwell & Griffin, 1973; Emert et al., 1974; Berghem et al., 1975) leaves no doubt that the observed activities of Ex-1 are those of a type of a type of true cellulase. Quantitative comparison of the activities of Ex-1 in catalyzing the hydration of cellobial vs. the hydrolysis of cellulose or cellodextrin is difficult to make at present. The enzyme's affinity for cellobial (K_m = 31 mM; Figure 1) clearly is much lower than, for example, its affinity for cellopentaose (K_m = <1 mM; Kanda et al., 1978). On the other hand, it is uncertain whether the observed fairly high rate of hydration of 40 mM cellobial (v = 0.23 μmol min^{-1} mg^{-1}), compared to that of hydrolysis of 0.25% Avicel insoluble cellulose (v = 0.057 μmol min^{-1} mg^{-1}; Kanda, 1978), reflects more than a difference in reaction conditions. Conceivably, cellobial might be utilized more rapidly than cellulose under identifical assay conditions since its hydration yields no byproduct, whereas cellobiose cleavage from the end of a cellulose chain leaves an unproductively bound residue that must depart from the enzyme before the next catalytic event.

That *A. niger* endocellulase per se should be able to act on a dimeric substrate to form 2-deoxycellobiose is supported by observations of the ability of other highly purified endocellulases to hydrolyze dimeric substrates to form cellobiose. The En-1 endocellulase of *I. lacteus* has been shown to hydrolyze *o*-nitrophenyl β-cellobioside and to utilize it in cellobiosyl-transfer reactions (Kanda et al., 1983); in addition, *I. lacteus* endocellulases (Kubo & Nisizawa, 1984; T. Kanda and Hehre, unpublished results) and *Trichoderma viride* endocellulase II-B (G. Okada and E. J. Hehre, unpublished results) have been found to catalyze the rapid hydrolysis of β-cellobiosyl fluoride to form cellobiose.

In order to define the mechanistic functioning of the present cellulases in catalyzing cellobial hydration and in catalyzing cellulose and cellodextrin hydrolysis, use is made of information available on the catalytic elements of glycosidases and on how they may function in reactions with glycosidically linked substrates. Previous workers (Legler, 1963, 1973; Quaroni et al., 1974, 1976; Lalegerie et al., 1982) have obtained considerable evidence for the presence of carboxyl groups in the active sites of various glycosidases, including cellulases from *A. niger* and an *Oxyporus* species (Legler & Bause, 1973),

Scheme I



and have postulated possible catalytic mechanisms for these enzymes on the basis of the observed disposition of two carboxyl groups in the active site of lysozyme (Blake et al., 1967). The mechanism of hydrolysis by the latter is generally believed to involve the cooperative functioning of these two carboxyl groups, such that one (located near the glycosidic oxygen atom of substrate) acts as a general acid to protonate that oxygen atom while the other, a carboxylate anion located on the other side of the center of substitution, acts as a charge-stabilizing group or nucleophilic-specific base (Blake et al., 1967; Phillips, 1967; Johnson et al., 1968). Despite a lack of knowledge about the nature of the catalytic groups of the present *I. lacteus* and *A. niger* cellulases, an analogous mechanism for the reactions they catalyze with cellulosic substrates appears to be a reasonable assumption. The β-1,4-linked compounds of D-glucose hydrolyzed by the cellulases are close analogues of the β-1,4-linked compounds of 2-(*N*-acetylamino)-2-deoxy-D-glucose hydrolyzed by lysozyme; and the hydrolytic reactions catalyzed by the present cellulases, providing overall retention of (β) configuration (Kanda et al., 1978; Okada, 1985), have the same steric course as those catalyzed by lysozyme.

The catalytic mechanism envisioned for the hydrolysis of cellulose by these cellulases is shown in Scheme IA. The illustrated mode of substrate binding at the active center provides for the cleavage of cellobiose from the nonreducing end of the molecule, a major type of reaction catalyzed by the Ex-1 enzyme; it also allows the proposed hydrolysis mechanism to be readily compared with that for the hydration of cellobial (Scheme IB). With other binding modes, the mechanism shown in Scheme IA would serve for the hydrolysis of interior glycosidic linkages, predominantly catalyzed by random-type cellulases such as the endo enzyme of *A. niger*. One carboxyl group, in the protonated state, is assumed to be located above the glycosidic oxygen atom and to act as a general acid to protonate the oxygen atom. This corresponds to Legler and Bause's (1974) conclusion, based on the inhibitory actions of epoxyalkyl cellobiosides on carboxymethylcellulose hydrolysis by *A. niger* and *Oxyporus* sp. cellulases, that a carboxyl group located above the glycosidic oxygen is the essential acid catalyst. The protonation step is pictured as leading to formation of a transient carbonium ion–enzyme complex that is stabilized by a carboxylate anion located below the C-1 center of substitution. A second step would effect breakdown of this complex by a directed attack of water at C-1 to form cellobiose of β-configuration as reported for the present enzyme preparations.

The mechanism proposed for the hydration of cellobial catalyzed in D_2O (Scheme IB) is similar to that reported for the hydration of D-glucal by sweet almond β-glucosidase (Hehre et al., 1977). We assume that cellobial binds at the active center of cellulase in the same way and with the same orientation to the catalytic groups as the terminal cellobiosyl moiety in a substrate undergoing hydrolysis to form cellobiose; the hydroxyl groups of cellobial are spatially disposed like those of the cellobiosyl moiety. The observed direction of protonation (deuteration) of cellobial is pictured as due to specific acid catalysis by an enzymic carboxyl group located near and below the double bond of the D-glucal moiety. This would be expected to lead to the formation of an incipient 2-deoxycellobiosyl oxycarbonium ion since D-glucal is a cyclic vinyl ether. Assuming this intermediate was stabilized ionically, stereospecific general base catalyzed hydration involving a suitably disposed carboxylate group of the enzyme would then yield the observed product β-D-glucopyranosyl(1→4)-2-deoxy-β-D-glucose-2(*e*)-*d*. Alternatively, collapse of the glycosyl carbonium ion might occur with production of a covalent 2-deoxycellobiosyl–enzyme intermediate, which would be subject to nucleophilic attack.

The assumed arrangement of two carboxyl groups on opposite sides of the active center of cellulase, as in lysozyme, gains credibility as a model of the catalytic elements of cellulase by its ability to account for the features of cellobial

hydration as well as those of cellulose hydrolysis. This ability, however, could not exist unless the catalytic groups are functionally flexible beyond the requirements of the principle of microscopic reversibility. Thus, in the first step of catalysis, the two carboxyl groups' specific functions in attacking the glycosidic linkage of a cellulosic substrate (Scheme IA) are reversed in attacking the double bond between C-1 and C-2 of cellobial (Scheme IB). Although subsequent steps in the catalysis of cellulose hydrolysis and cellobial hydration may possibly be identical, each enzyme uses two different mechanisms in effecting the stereochemically distinct reactions catalyzed with the two different types of substrate.

The present demonstration of the latter point with cellulases adds support for the concept (Hehre et al., 1979) that glycosylases in general have catalytic groups that are functionally flexible beyond the requirement of the principle of microscopic reversibility. Recent studies of glycosylation reactions catalyzed with nonglycosidic substrates by other enzymes [for example, by β-galactosidase (Lehmann & Schlesselmann, 1983), trehalase (Hehre et al., 1982; Kasumi et al., 1986), and maltose phosphorylase (Tsumuraya et al., 1984)] likewise provide new evidence in support of that concept. With catalytic group flexibility as a likely characteristic of many if not all glycosylases, an enzyme of this type may no longer be safely assumed to act on all substrates by a single mechanism as implied in the commonly used expression "the mechanism of an enzyme".

Registry No. I, 490-51-7; II, 99809-92-4; cellulase, 9012-54-8; hexa-*O*-acetylcellobial, 67314-36-7; glycosylase, 9032-92-2.

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