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Direct ^{1}H NMR evidence for conversion of β-D-cellobiose to cellobionolactone by cellobiose dehydrogenase from *Phanerochaete chrysosporium*

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Abstract

The α- and β-anomers of D-cellobiose were resolved by ^{1}H NMR spectroscopy. Addition of cellobiose dehydrogenase purified from the white-rot *P. chrysosporium* led to selective conversion of β-D-cellobiose. The product was identical to cellobionolactone as synthesized from Ca-cellobionate. Overnight incubation of the product led to an altered NMR spectrum, which was also obtained by incubation of cellobionolactone. The new spectrum matched that for Ca-cellobionate. The instability of cellobionolactone explains the detection of cellobionic acid as product in earlier studies.

Key words: Anomeric specificity; Cellobionolactone; D-Cellobiose; Cellobiose dehydrogenase; Lactone hydrolysis; *Phanerochaete chrysosporium*

1. Introduction

The white-rot fungi are unique in their ability to degrade lignin. Their biotechnological applications include pulp bleaching for the paper industry, processing of lignocellulosic wastes and bioremediation of recalcitrant pollutants [1]. The realization of this potential requires an understanding of the enzyme mechanisms involved. Much of the recent research has concerned peroxidases (ligninase, Mn-peroxidase) and hydrolases (e.g. cellulases) but it is becoming increasingly evident that other enzymes are also important.

In 1974, Eriksson et al. [2] observed that cellulose breakdown by culture filtrates of *Phanerochaete chrysosporium* was doubled in rate by the presence of oxygen. This suggested that in addition to cellulases, an oxidative enzyme was also present. An enzyme capable of oxidizing D-cellobiose and higher cellodextrins with molecular oxygen as electron acceptor was purified by Ayers et al. [3] and given the initial name of cellobiose oxidase. This name has recently been modified to cellobiose dehydrogenase (CDH), since reduction of quinones, semiquinone radicals and Fe(III) is much faster than that of oxygen [4,5]. Putative roles in lignin degradation and production of Fenton's reagent are discussed in recent reviews [5,6].

The substrate range for oxidation by CDH extends from D-cellobiose to solid cellulose [3,7]. The initial research by Ayers et al. [3] indicated that cellobionic acid is the product of cellobiose oxidation (see Scheme 1), since glyoxylic acid was detected after oxidative cleavage with periodate. Similar results have been reported for CDH from *Thielavia heterothallica* (*Sporotrichum thermophile*) and *Chaetomium cellulolyticum* [8,9]. However, earlier experiments with cellobiose:quinone oxidoreductase from *P. chrysosporium* had demonstrated the formation of cellobionolactone, determined as a hydroxamic acid derivative [10]. Cellobiose:quinone oxidoreductase lacks the haem prosthetic group of CDH but has an identical range of carbohydrate substrates (see [11] for a discussion of their relationship). This similarity led Ayers et al. [3] to raise the possibility that CDH might give the lactone as initial product, with cellobionic acid being formed by a subsequent hydrolysis. More recently, many papers have stated that CDH converts cellobiose into cellobionolactone (e.g. [4,12,13]). As far as we are aware, our results provide the first direct investigation.

It is clear that CDH modifies the reducing end (C-1) of its substrate. The hemiacetal hydroxyl group in this position has one of two configurations: axial (α-anomer) or equatorial (β-anomer), as shown in Scheme 1. The specificity of CDH for one anomer relative to the other has never previously been considered. It is revealed here by ^{1}H NMR spectroscopy, in experiments analogous to those used for purified cellulases to determine which anomer is formed as product [14,15].

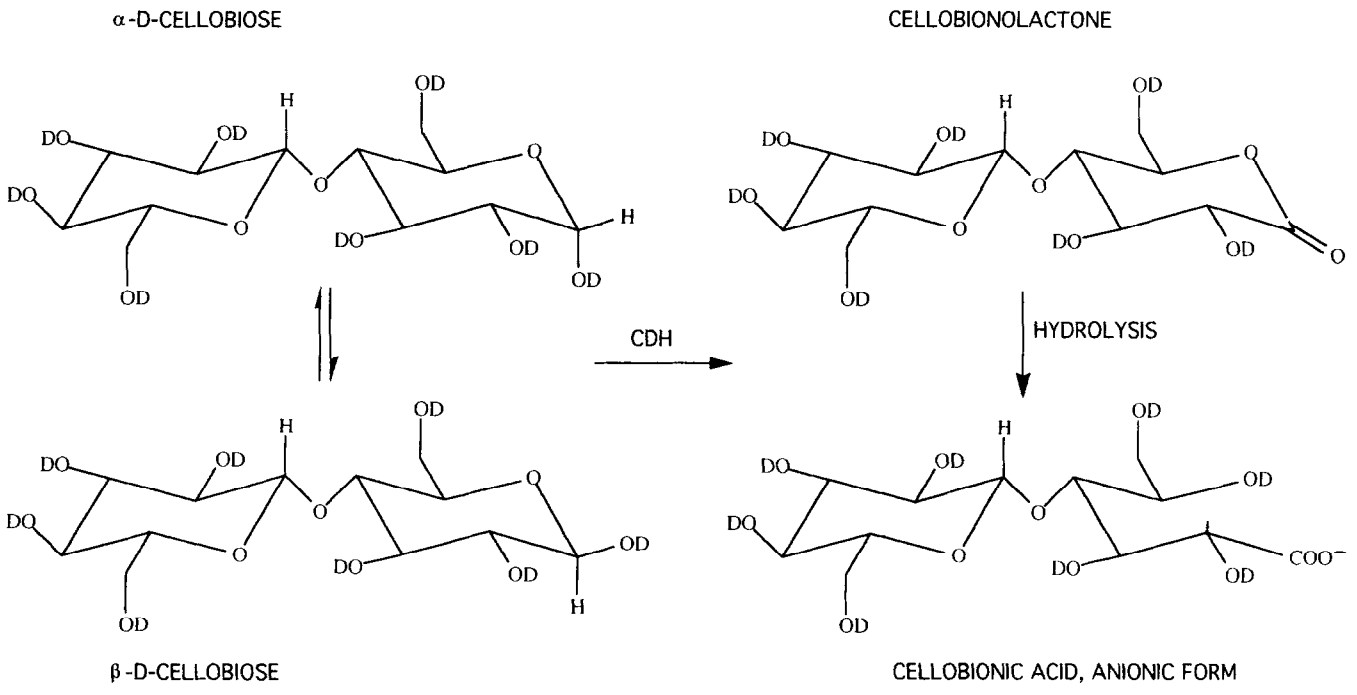
2. Materials and methods

Growth of *P. chrysosporium* and purification of cellobiose dehydrogenase were as described in [16]. Water in the CDH preparation was exchanged for D_2O by four cycles of concentration from 500 μl to 30 μl in a vacuum evaporator.

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Abbreviation: CDH, cellobiose dehydrogenase.

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Scheme 1. Potential reactants and products for D-cellobiose conversion by CDH. The C-1 protons are indicated. Hydroxyl groups are shown after exchange with D_2O.

The preparation of cellobionolactone was based on [17]. Calcium cellobionate (1 g) was dissolved in deionized water and passed through a cation exchange column in the H^+ form (Amberlite IR 118-H, 3 ml), the column being washed with 50 ml water. The cellobionic acid was converted to the lactone by removal of water; the eluent was concentrated at 50°C in a rotary evaporator and dried by three successive evaporations in 6 ml of dry 2-methoxyethanol and toluene (2:1, v/v). The yield was 350 mg. An IR spectrum showed a major band at 1732 cm^{-1}, corresponding to the C=O stretch of the lactone.

Deuterium oxide was of 99.96 atom% D stated isotopic purity. Acetic acid (8.25 mM) and Na-acetate (2.5 mM) were added, giving a calculated pD of 4.7 assuming pD = 5.2 for an equimolar mixture [18]. D-Cellobiose was dissolved immediately before use. Potassium ferricyanide (6 mM) was added as electron acceptor and was present in all controls.

^1H NMR spectra of 0.5 ml samples in 5 mm NMR tubes were obtained at 26°C using the Jeol alpha 500 MHz spectrometer of the Bristol University Molecular Recognition Centre. 64 free induction decays (FID) were collected in 16 K complex data points. The FID were multiplied by a mild Gaussian window function before Fourier transformation. The residual water proton signal was suppressed by irradiation during the relaxation delay. Spectra were referenced relative to the chemical shift of the anomeric hydrogen of α-D-cellobiose at 5.23 ppm [19].

All materials were from Sigma, except for 2-methoxyethanol, D_2O and the Amberlite cation exchanger which were from Aldrich.

3. Results

In the spectra illustrated here, only C-linked protons are observed because hydroxyl groups give rapid H/D solvent exchange [20]. The protons of especial interest are those at C-1, for which cellobiose has one on each ring (see Scheme 1). Their signals are shifted downfield (higher δ) by the presence of two C_α oxygen atoms. They are also unique in coupling to only one other proton (at C-2) and hence give distinctive doublets. Fig. 1a shows a ^1H NMR spectrum for D-cellobiose in D_2O. This includes doublets for the α-anomeric and β-anomeric protons at $\delta = 5.23$ and 4.67, respectively, while the internal anomeric proton gave a doublet at $\delta = 4.52$ [19].

The other three spectra in Fig. 1 were taken after addition of CDH and show a progressive decline in the β-anomeric and internal-anomeric signals. The signal for the α-anomeric proton meanwhile increased slightly in intensity. This slight rise can be explained by mutarotation; the freshly dissolved cellobiose was predominately the β-anomer, while the equilibrium proportions are $\beta:\alpha = 63:37$ [21]. The time constant for uncatalysed mutarotation of D-cellobiose at 26°C is 43 min [22,23]. The conclusion is that CDH is specific for β-D-cellobiose as substrate for oxidation.

It is evident that many new signals appeared after CDH was added and increased in size as the cellobiose was oxidized. In Fig. 2, a spectrum taken 32 min after addition of CDH is compared with one for cellobionolactone. The cellobionolactone spectrum includes the following: $\delta = 4.58$, doublet; $\delta = 4.44$, doublet with fine structure; $\delta = 4.28$, doublet; and $\delta = 4.15$, triplet. All these features are matched by signals that developed as cellobiose was oxidized by CDH. The $\delta = 4.58$ doublet must be from the internal anomeric proton, while the others can be tentatively assigned to the protons at C-4, C-2, and C-3 of the lactone ring, respectively (M.R. Sinnott, personal communication). Thus ^1H NMR spectra

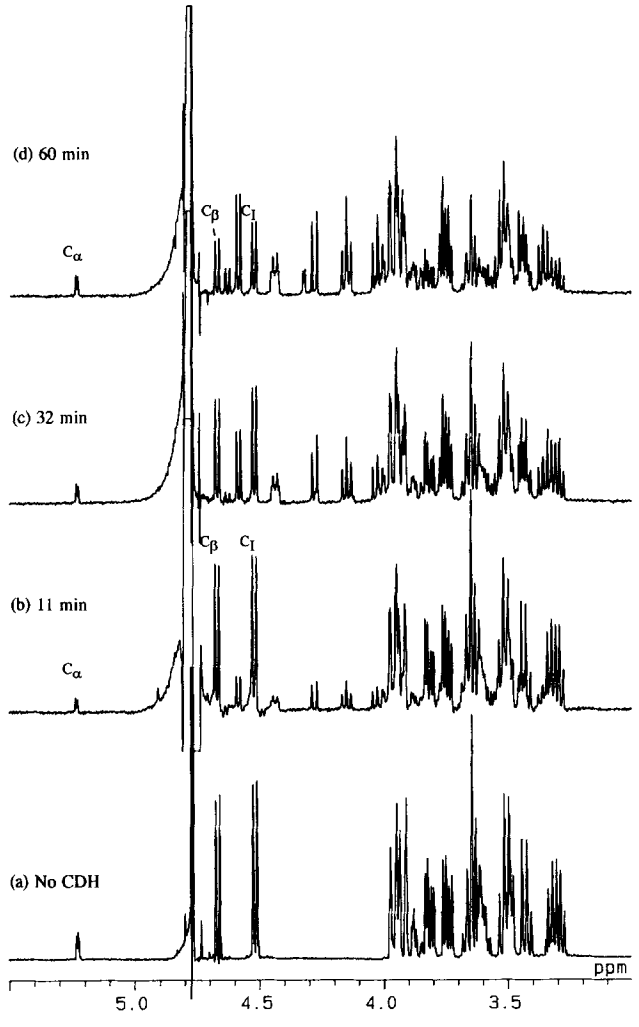


Fig. 1. NMR spectra for oxidation of cellobiose by cellobiose dehydrogenase. D-Cellobiose was present at 3 mM, in acetate buffer plus K-ferricyanide (6 mM). (a) Reference spectrum without CDH. (b) 11 min after addition of CDH (0.29 μM), using a different solution of D-cellobiose. (c,d) As (b), but 32 and 60 min after CDH addition, respectively. The symbols C_α, C_β and C_I indicate the signals for α-anomeric, β-anomeric and internal C-1 protons in D-cellobiose.

provide conclusive evidence that cellobionolactone is formed as the initial product.

The reference spectrum for cellobionolactone (Fig. 2b) includes a few signals that are not matched by cellobiose plus CDH. A strong peak at $\delta = 3.39$ is attributable to residual 2-methoxyethanol [24]. The minor peaks at $\delta = 4.61$ and 4.23 are a sign of slight hydrolysis (see below) while the minor peak at $\delta = 4.38$ may indicate an unidentified impurity.

Cellobionolactone is not completely stable in dilute aqueous solution. The time constant for spontaneous hydrolysis is 61 min at 26°C [25]. At the pH of these experiments, the predominant product will be the cellobionate anion (pK = 3.5 in water [25]). Fig 3a presents an NMR spectrum for Ca-cellobionate dissolved in acetate buffer, while the spectra in Fig 3b and c are for preparations of cellobionolactone and CDH plus cellobiose that had been left overnight to equilibrate. The three spectra show a high degree of similarity, including a doublet at $\delta = 4.63$, attributable to the internal anomeric proton. The position of the C-2 proton's signal will depend on the ratio of cellobionic acid to cellobionate; cf. $\delta = 4.48$ for D-gluconic acid, 4.16 for D-gluconate [26]. All three spectra show a doublet in this region, with slight differences in δ value (from $\delta = 4.28$ for cellobiose treated with CDH to $\delta = 4.19$ for Ca-cellobionate) probably reflecting differences in pD. The signals in the cellobionolactone preparation that were attributed above to impurities ($\delta = 3.39$ and 4.38) were unchanged by overnight incubation.

4. Discussion

The anomeric specificity is known for many enzymes that oxidize the reducing end of aldopyranoses, for example: D-arabinose dehydrogenase (α-D-arabinose); L-fucose dehydrogenase (β-L-fucose); D-galactose dehydrogenase (β-D-galactose); D-glucose dehydrogenase, D-glucose:D-fructose oxidoreductase, D-glucose oxidase and D-glucose-6-phosphate dehydrogenase (all β-D-glucose) [27–29]. In all cases the reactive anomer has an

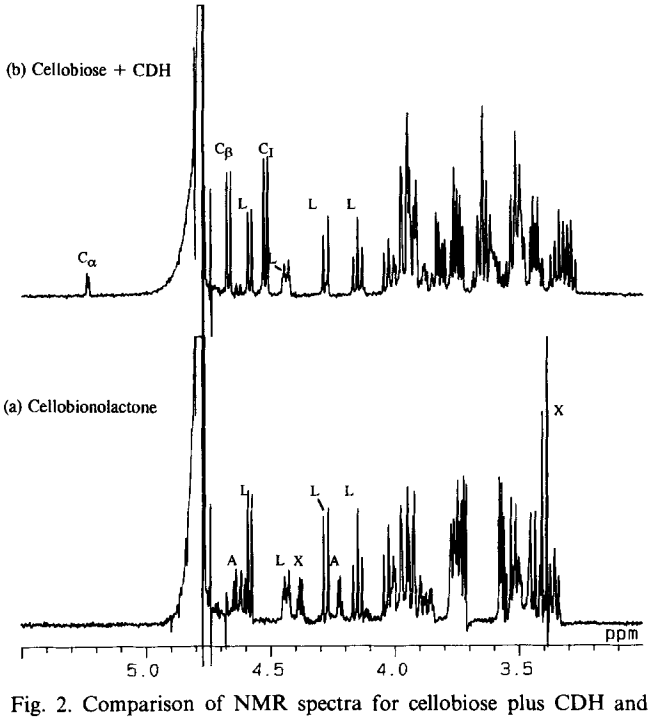


Fig. 2. Comparison of NMR spectra for cellobiose plus CDH and cellobionolactone. (a) D-Cellobiose (3 mM) plus CDH (0.29 μM), 32 min after addition of CDH. (b) Cellobionolactone (3 mM), freshly dissolved in D_2O. Acetate buffer and K-ferricyanide were present for both spectra. The symbols C_α, C_β and C_I are as in Fig. 1. Other signals discussed in the text are labelled as follows: L, lactone; X, impurities; A, cellobionate (cf. Fig. 3).

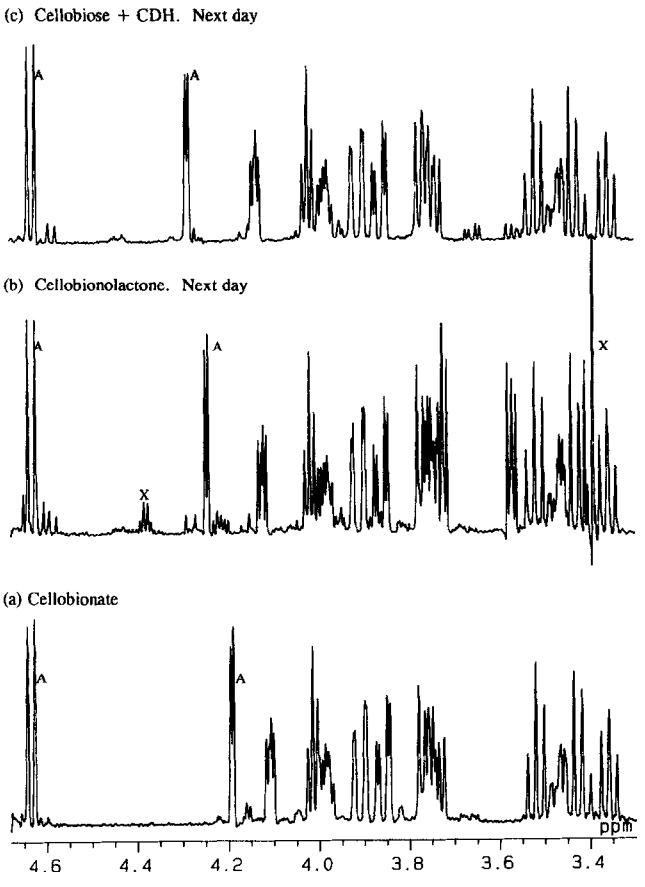


Fig. 3. Comparison of NMR spectra for cellobiose plus CDH at prolonged incubation and the cellobionate anion. (a) CDH plus cellobiose as in Fig. 2a, after overnight incubation at 20°C. (b) Cellobionolactone as in Fig 2b, after overnight incubation at 20°C. (c) Calcium cellobionate (3 mM), freshly dissolved in acetate buffer plus K-ferricyanide. Certain signals are labelled as in Fig. 2.

equatorial hydroxyl group; labelling conventions make this the β-anomer for hexoses and α-anomer for pentoses such as arabinose. Thus CDH conforms to the pattern. It might be thought that these enzymes have merely evolved to utilize the anomer that predominates at equilibrium. However Berkowitz and Benner [28] present a mechanistic argument; with an equatorial hydroxyl group the two oxygens attached to the anomeric carbon each bear a lone pair of electrons antiperiplanar to the departing 'hydride'.

Ayers et al. [3] proposed that CDH stimulates the rate of cellulose breakdown by prevention of 'snapback'. This role is based on the reversibility of the cellulase reaction. Thus a cellobiohydrolase cleaves cellobiose from the non-reducing end of a cellulase chain, but can also catalyse the condensation of cellobiose with existing chains [30]. Oxidative modification of cellobiose by CDH would make the hydrolysis irreversible. However, it is now clear that cellulases work by two contrasting mechanisms; some give retention of configuration and release the β-anomer while others give inversion and release the α-anomer [14,15]. Most organisms that are strongly cellulolytic contain cellulases in both of these categories, according to sequence data [31]. Our results imply that for inverting cellulases, mutarotation would be necessary before CDH could have any effect.

The turnover of CDH releases electrons which can be detected as a current or change in potential. This has led to interest in its use as a biosensor for determining cellodextrin concentrations or cellulase activity [32,33]. It has also been used in a spectrophotometric assay for lactose in milk [34]. Our results indicate that in a short time scale, such assays will be restricted to the β-anomer. The α-anomer can only be detected after mutarotation has occurred. It may be desirable to increase the rate of mutarotation, by adding mutarotase or an acid/base catalyst.

The formation of the lactone as product has physiological implications. Lactones, including cellobionolactone, act as inhibitors of cellulases and β-glucosidase [35]. Cellobionolactone is a potent inducer of cellulase synthesis in *Trichoderma reesei* [36]. The lactone concentration will be limited not only by spontaneous hydrolysis but also by lactonase, which has been detected in a wide variety of commercial cellulases and also reported from *P. chrysosporium* [10,37].

Our findings raise questions about rates of anomer interconversion, lactone formation and lactone hydrolysis during growth of *P. chrysosporium* and other lignocellulolytic fungi. The resolution of such compounds by ^{1}H NMR should prove valuable in further research.

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