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Stereoselective oxidation of protected inositol derivatives catalyzed by inositol dehydrogenase from *Bacillus subtilis*†

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Inositol dehydrogenase (EC 1.1.1.18) from *Bacillus subtilis* is shown to have a nonpolar cavity adjacent to the active site, allowing racemic protected inositol derivatives such as 4-*O*-benzyl-*myo*-inositol to be recognized with very high apparent stereoselectivity.

myo-Inositol dehydrogenase (IDH), EC 1.1.1.18, is a bacterial enzyme that catalyzes the selective oxidation of the (axial) hydroxyl group attached to carbon-2 of *myo*-inositol to a ketone with concomitant conversion of NAD^+ to NADH, as shown in Fig. 1. The reaction allows the organism to grow on inositol, which is abundantly available in soil, as its sole carbon source. The gene encoding this enzyme has been identified in many environmental bacteria, but only in the case of IDH from *Bacillus subtilis* has the catalyzed reaction been studied in detail.[1] Among the findings of that work, it was reported that IDH catalyzed the oxidation of α-D-glucopyranose and D-xylose to the corresponding 1,5-lactones, but that *scyllo*-inositol and β-D-glucopyranose (in which all the substituents are equatorial) were not substrates. This indicates that an axial hydroxyl group is a required feature of the substrate, but that other aspects of the structure may vary. Many other common monosaccharides and alditols were also excluded as substrates, suggesting that the IDH active site does discriminate against most variations in structure. For example, galactose and mannose, containing an additional axial hydroxyl group, were not oxidized, nor was glucose-6-phosphate a substrate. Our laboratory has chosen to probe the active site of *B. subtilis* IDH further in order to learn more about its catalytic properties, and to discover if such an enzyme could be used as an efficient means of separating enantiomers of *myo*-inositol derivatives, providing a source of optically active cyclitols. Inositol derivatives are ubiquitous natural products, and the enzymes that manipulate them are therapeutic targets for the treatment of several illnesses.[2] The efficient synthesis of optically active inositols is by no means routine.[3]

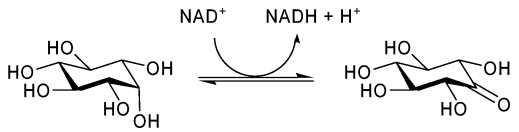

Fig. 1 Oxidation of *myo*-inositol to *scyllo*-inosose by inositol dehydrogenase.

The sequence of the gene encoding IDH, *iolG*, has been reported previously.[4] PCR-amplification from *B. subtilis* 168 (trpC2) genomic DNA using primers based on the reported sequence resulted in an amplified sequence which differed by one nucleotide from the coding region reported. This was true of two separate PCR experiments, performed on different template preparations, by different individuals, suggesting that the sequence of our template is accurately reflected in the clone. The difference, a thymine at nucleotide 434 rather than the reported adenine, results in an amino acid change at position 145, from asparagine to isoleucine. Inspection of apparently homologous sequences, such as those found in the cluster of orthologous groups COG0673[5] indicates that the amino acid corresponding to position 145 of the *iolG* gene product is always a branched, hydrophobic residue such as leucine, isoleucine or valine. We therefore believe this sequence represents that of wild-type IDH, and it has been deposited in GenBank (accession no. AY676876). The gene was ligated into pET-28b and expressed in *E. coli* BL21(DE3). Purification of IDH bearing an *N*-terminal histidine tag by Ni^{2+}-affinity using a gradient of increasing imidazole concentration resulted in protein that was determined to be homogeneous by SDS-PAGE analysis. Electrospray mass spectrometry indicated a molecular mass of 40382.0; the predicted exact mass based on our sequence is 40382.76. The resulting IDH, assayed by observing the appearance of NADH at 340 nm, was active without removing the His tag. The kinetic constants for the oxidation of *myo*-inositol in the presence of recombinant enzyme, shown in Table 1, are in good agreement with those reported previously for the native enzyme. Ramaley *et al.*[1] showed the IDH-catalyzed reaction to follow an ordered sequential Bi Bi mechanism, with $K_m^{NAD} = 0.23$ mM and an apparent Michaelis constant for inositol $K^{App} = 18$ mM.‡

Table 1 Kinetic constants of IDH-catalyzed oxidations[a]

Compound	K^{App}/mM	V^{App}/μmol min^{-1} mg^{-1}	$(V/K)_{rel}$/%
myo-Inositol	18 ± 2	8 ± 1	100
1	8 ± 1	0.8 ± 0.1	26
2	7 ± 1	0.8 ± 0.1	30
3	4 ± 1	0.9 ± 0.1	51
4	3 ± 1	0.8 ± 0.1	58
5	44 ± 5	1.6 ± 0.1	8
6	13 ± 2	0.7 ± 0.1	12
7	—	Trace activity detected	—
Melibiose	57 ± 6	2.0 ± 0.5	8
Gentiobiose	—	Trace activity detected	—

[a] Conditions: 100 mM Tris–HCl, pH 9.0, 25 °C. $[NAD^+] = 0.5$ mM.

The previous report that IDH can oxidize D-glucose[1] led us to propose that IDH might tolerate small substituents on inositol at the position analogous to C-6 of D-glucose. For example, 1L-4-*O*-methyl-*myo*-inositol is approximately isosteric with α-D-glucopyranose, as indicated in Fig. 2. We confirmed

† Electronic Supplementary Information (ESI) available: experimental details: molecular biology, enzyme kinetics, HPLC conditions, and synthesis and characterization of all synthetic substrates. See http://www.rsc.org/suppdata/ob/b4/b417757f/

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the previous results with D-glucose and D-xylose, and also tested L-glucose and L-xylose, but could observe no activity, suggesting a high degree of stereoselectivity, *i.e.* one 'side' of the active site can tolerate variations in structure, but the other 'side' recognizes equatorial hydroxyl groups. The 4/6-position is conveniently alkylated, sulfonylated or phosphorylated using the 1,3,5-monoorthoformate route pioneered by Kishi.[6] Briefly, *myo*-inositol monoorthoformate is formed by an acid-catalyzed reaction of triethyl orthoformate with inositol. This product is alkylated or acylated with the appropriate electrophile, and the protecting group(s) subsequently removed. While this process results in good yields and can be run on a large scale, it does result in racemic products. We have used these methods to synthesize an array of substituted inositols, shown in Fig. 2.†

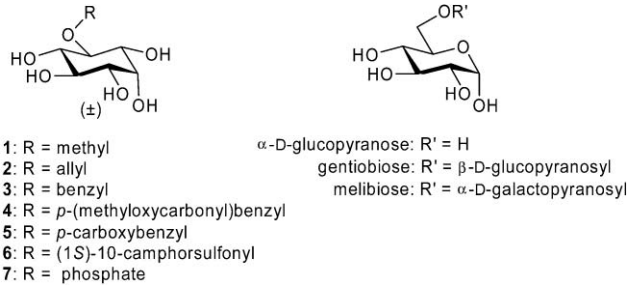

Fig. 2 Racemic 4-*O*-substituted *myo*-inositol derivatives (the 1L-enantiomer is drawn) and D-glucose derivatives oxidized by IDH.

4-*O*-Methyl-*myo*-inositol was a substrate of IDH, as we had predicted. When we repeated our experiments with larger substituents, we were surprised to find that solubility was the primary limitation to suitability as a substrate. Even a very bulky substituent, such as the (1*S*)-10-camphorsulfonyl group, was tolerated by the enzyme. All showed normal hyperbolic dependence of rate on substrate concentration. Disappointingly, when inositol 4-phosphate (**7**) was the substrate only trace activity could be detected, perhaps unsurprising given that no activity could be observed with glucose-6-phosphate. However, we were particularly encouraged that substituents commonly used as protecting groups in multi-step syntheses, such as the allyl and benzyl groups, could be incorporated while maintaining enzymatic activity. The data in Table 1 show that a more favourable Michaelis constant is observed for several substrates relative to the natural substrate, indicating an enhanced enzyme–substrate interaction in the ground state. The results using compounds **4** and **5** show that while a negatively charged substituent can be tolerated in the active site (when distant from the inositol moiety), the Michaelis constant increases by an order of magnitude.

The equilibrium of the IDH reaction favors the inositol rather than the inosose form. To drive the reaction to completion, the reaction was performed in the presence of lactate dehydrogenase and an excess of pyruvate. This allowed a catalytic amount of cofactor to be used, since it is continually recycled. HPLC analysis of the reaction mixture using a reverse-phase column indicated that the amount of substrate had decreased by 50%. Using a cyclodextrin-based chiral HPLC column, we found that racemic **3** could be resolved. After 72 h of enzymatic reaction, one of the peaks was completely consumed. The remaining substrate was isolated, and a specific rotation of +6 could be measured. This is the value reported previously for 1D-4-*O*-benzyl-*myo*-inositol.[7] When this isomer was re-subjected to the IDH assay, no activity could be observed. The optically active 1L-**3** synthesized in our laboratory[7,8] co-elutes with the reactive isomer when analyzed by HPLC, and is a substrate for IDH. Note that we synthesized racemic **3** in 73% yield starting from *myo*-inositol, and could obtain the 1D-isomer quantitatively using IDH. Chemical synthesis of optically active **3** by published methods resulted in an overall yield of less than 2%.

The presence of a relatively nonpolar cavity adjacent to the active site of IDH suggests that the enzyme may have a role in the oxidation of other natural products *in vivo*. Alternatively this may indicate that IDH has evolved from an enzyme that recognizes more complex structures, and has retained some of the characteristics of the progenitor. Speculation as to the evolutionary origin of this active site led us to investigate disaccharides as possible substrates. We chose gentiobiose [6-*O*-(β-D-glucopyranosyl)-D-glucopyranose] and melibiose [6-*O*-(α-D-galactopyranosyl)-D-glucopyranose] because they are roughly isosteric with 1L-4-*O*-substituted inositols, and were readily available. Neither 'gentiobiose dehydrogenase' nor 'melibiose dehydrogenase' are reported in the literature, however melibiose transporter and hydrolase genes are apparently present in *B. subtilis*.[4b] Both compounds were oxidized in the presence of IDH, although the activity observed with gentiobiose was very low. The apparent Michaelis constant of the melibiose reaction was within experimental error of that previously reported for α-D-glucopyranose. Initial experiments with isomaltose [[6-*O*-(α-D-glucopyranosyl)-D-glucopyranose] show activity similar to that observed with melibiose suggesting the α(1,6) linkage results in a better fit in the IDH active site.

In summary, we have probed the active site of IDH, revealing a hydrophobic cavity that allows the recognition and oxidation of 1L-4-*O*-substituted *myo*-inositols bearing large hydrophobic groups. This property allows resolution of racemic mixtures of inositol derivatives, including those blocked with cleavable protecting groups that may be used in the asymmetric synthesis of more complex inositol derivatives. The relatively low substrate selectivity and high stereoselectivity of IDH suggest it is well-suited for development as a tool for synthetic applications. The oxidation of disaccharides by IDH suggests the enzyme may fulfil such a role *in vivo*, or it is homologous with an as-yet-unidentified disaccharide dehydrogenase. Understanding the relationship between enzyme structure and substrate recognition will help in assigning function to putative oxidoreductases whose metabolic role cannot be predicted based on our current knowledge.

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Notes and references

‡ Determined at $[NAD^+] = 0.5$ mM. Reactions were fit to the equation $v = V^{App}[S]/(K^{App} + [S])$ where S is the varied substrate and V^{App} is the maximal velocity at the concentration of the fixed substrate. In an ordered sequential Bi Bi mechanism, $K^{App} = K_m^{inositol}(K_i^{NAD} + [NAD^+])/(K_m^{NAD} + [NAD^+])$, where K_i^{NAD} is the product inhibition constant for the reverse process (binding of NAD^+ to free enzyme).[9] Using our data, we calculate $K_i^{NAD} = 5$ mM, and $K_m^{inositol} = 1$ mM.

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